THE JOURNAL

OF

PHARMACOLOGY

AND

EXPERIMENTAL THERAPEUTICS

FOUNDED BY JOHN J. ABEL

OFFICIAL PUBLICATION
OF THE AMERICAN SOCIETY FOR PHARMACOLOGY AND
EXPERIMENTAL THERAPEUTICS INCORPORATED

VOLUME 95

1949

BALTIMORE, MARYLAND

CONTENTS

Number 1, January, 1949

Number 2, February, 1949

NUMBER 4, APRIL, 1949

MECHANISM OF DIBENAMINE PROTECTION AGAINST CYCLOPROPANE-EPINEPHRINE CARDIAC ARRHYTHMIAS[1]

MARK NICKERSON AND GEORGE M NOMAGUCHI

Department of Pharmacology, University of Utah School of Medicine, Salt Lake City, Utah

Received for publication September 14, 1948

The demonstration of the highly specific and effective adrenergic blocking action of Dibenamine (N,N-dibenzyl β chloroethylamine) (1, 2) suggested that it might be effective in preventing serious cardiac arrhythmias produced by epinephrine in the presence of cyclopropane and other hydrocarbons The protection against epinephrine cyclopropane arrhythmias afforded by Dibenamine proved to be almost complete (fig 1 and table I) even when massive doses of epinephrine (1000 μgm /kgm) were injected intravenously (3, 4)

However, Dibenamine does not prevent the chronotropic and positive inotropic effects of epinephrine on the mammalian heart (2, 5), consequently the basis for a direct protection of the myocardium is obscure and protection as a result of peripheral vascular actions of the drug must be considered

Possible modes of action include (1) some direct effect on the myocardium, (2) prevention of the epinephrine induced rise in systemic blood pressure and of the resultant increase in left ventricular pressure, or (3) prevention of reflex vagal activity by abolishing the pressor response to epinephrine The following experiments were undertaken in an effort to distinguish between these possibilities

METHODS Dogs were administered sufficient thiopental sodium intravenously (usually 15–20 mgm /kgm) to allow passage of an endotracheal tube A 30 per cent cyclopropane 70 per cent oxygen anesthetic mixture was then administered by means of a closed system with CO_2 absorption A total gas flow of 1000 cc /minute was maintained to allow frequent flushing of the 3 liter rebreathing bag, and analysis showed the cyclopropane concentration in the bag to vary between 28 and 31 per cent This procedure maintained the animals in plane 3 anesthesia (partial to complete intercostal paralysis) After opening the pleura or at any time spontaneous respiratory exchange was markedly reduced, oxygenation was maintained by intermittent pressure on the rebreathing bag All tests were performed after 30 to 80 minutes of cyclopropane anesthesia to allow for complete equilibration and yet to avoid the "adrenolytic" effect of longer exposures to cyclopropane (6)

After equilibration to the anesthetic mixture had occurred, a standard challenge dose of 10 μgm /kgm epinephrine in 5 cc of 0 9 per cent NaCl solution was injected intravenously over a period of 50 seconds By means of a thermo writing oscillograph, standard limb lead electrocardiograms were recorded continuously during the injection of epinephrine and until the heart returned to its normal rate and rhythm after the injection In most cases three leads were recorded simultaneously

---

[1] This investigation was aided by a grant from Givaudan Delawanna, Inc The adrenergic blocking agents employed were kindly supplied by Dr William Gump of this company N Dibenzyl beta chloroethylamine is now being distributed for investigational use by the Smith, Kline and French Laboratories Philadelphia Pennsylvania under their trade mark Dibenamine

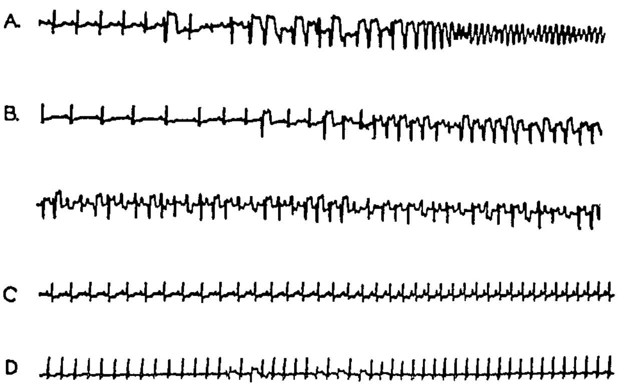


FIG 1 DIBENAMINE PROTECTION AGAINST CYCLOPROPANE-EPINEPHRINE ARRHYTHMIAS ALL ELECTROCARDIOGRAMS LEAD II

A Control dog showing ventricular premature contractions, tachycardia and fibrillation

B C ... ith survival

... Epinephrine produced a

... and only 5 abnormal beats in dog D

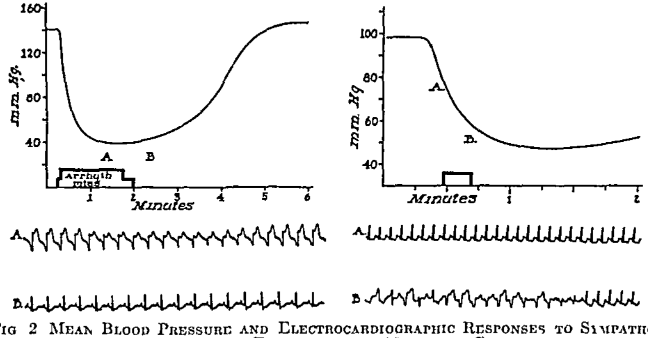


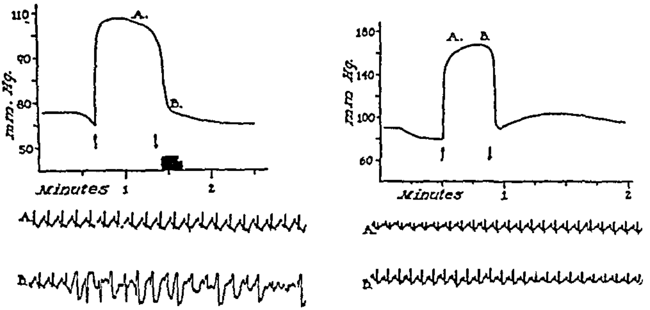
FIG 2 MEAN BLOOD PRESSURE AND ELECTROCARDIOGRAPHIC RESPONSES TO SYMPATHOMIMETIC AMINES IN DOGS EQUILIBRATED TO 30 PER CENT CYCLOPROPANE

Left Intravenous injection of 100 μgm /kgm epinephrine in animal pretreated with 5 mgm /kgm #186

Right Intravenous injection of 10 μgm /kgm Isuprel (N isopropyl nor epinephrine) in control animal Injections begun at zero time, periods of cardiac arrhythmias indicated by heavy rectangles Correlation of blood pressure and lead II electrocardiograms indicated at A and B

amine (15–20 mgm./kgm.) or #186 (10 mgm./kgm.) just adequate to prevent cardiac arrhythmias when the standard test dose of epinephrine was administered, and then subjected to the additional stress of a rise in blood pressure produced by occlusion of the thoracic aorta. In 9 out of 12 animals treated in this manner it was possible to induce ventricular premature contractions and even ventricular tachycardia (5 animals) and fibrillation (3 animals) during such periods of aortic occlusion.

With larger doses of blocking agent, irregularities were more difficult to elicit and in 2 out of 6 animals given over 30 mgm./kgm. Dibenamine or 20 mgm./kgm. #186 it was impossible to produce ectopic beats even when the pressure was raised to 160–180 mm. Hg (fig. 4, right). Although no ectopic foci of impulse



F7i ', MEAN BLOOD PRESSURE AND ELECTROCARDIOGRAPHIC RESPONSES TO AORTIC OCCLUSION

Dogs, equilibrated to 30 per cent cyclopropane and injected intravenously with 10 .../kg ... epin... Correlation of blood pressure and lead II electrocardiogram ... oth animals pretreated with 30 mgm./kgm. Dibenamine. Irr... k rectangle. Aortic occlusion and release indicated by arrows.

formation developed in these dogs, the electrocardiograms usually showed some depression of the S-T segment during the period of aortic occlusion. This change may be interpreted as indicating a relative hypoxia induced by increasing the work required of the left ventricle.

It was found that severe irregularities were produced more readily by occluding the aorta at the time the injected epinephrine first reached the heart or shortly thereafter than at the end of the period of epinephrine injection, although the concentration of epinephrine reaching the heart must be maximum about 60 seconds after the beginning of the injection. This is clearly demonstrated by a comparison of early and late periods of aortic occlusion in the same group of animals. In 15 experiments, 20-second periods of occlusion starting 20 seconds after the beginning of epinephrine injection were characterized by irregularities occupying 53 ±8.6 per cent of the occlusion time. Similar periods of occlusion in the same animals starting at 50 seconds exhibited only 11 ±3.6

per cent irregularities. The average maximum pressures during occlusion in these two groups differed by only 7 mm. Hg. These figures differ by 4.3 times the standard error of the difference and therefore represent a significant variation in susceptibility to cardiac arrhythmias.

An additional indication of the greater tendency for the development of arrhythmias shortly after the epinephrine first reaches the heart is the fact that in three animals ventricular fibrillation was precipitated by aortic occlusion at 15 to 20 seconds. Ventricular fibrillation never followed occlusion later in the course of the epinephrine injection although all animals were tested with occlusion at 40 or 50 seconds before earlier occlusion was attempted.

The conclusion that the maximum tendency for the heart to develop arrhythmias occurs when injected epinephrine first reaches it is in agreement with our previous observations (4). When ventricular fibrillation occurs in unprotected dogs it begins an average of only 8 seconds after epinephrine first reaches the heart, and the few irregularities which do appear in standard tests on animals treated with Dibenamine or Priscol always occur in the same period.

It is conceivable that either the sudden increase in systemic arterial pressure, the final absolute pressure after aortic occlusion or a combination of these factors might be involved in the production of arrhythmias. To differentiate between these possibilities, coefficients of correlation of the increase in pressure and the absolute pressure during occlusion with the extent of irregularities (i.e., per cent of the occlusion period occupied by irregularities) were calculated. On the basis of 23 experiments with occlusion 20 seconds after the beginning of the epinephrine injection coefficients of correlation of -0.41 between pressure increase and irregularities and $+0.60$ between final pressure and irregularities were calculated. The clear difference between these two values is due to the fact that with high initial pressures the increase is usually smaller although the final absolute pressure is greater. The positive correlation between absolute pressure and arrhythmias is highly significant and indicates that the absolute level of systemic arterial pressure is the important factor in sensitizing the heart to epinephrine-induced irregularities. Changes in pressure appear to be of very limited if any importance.

A second characteristic of the role of systemic blood pressure in inducing cardiac irregularities is that the effect of elevated pressure develops slowly. Short periods (5–10 seconds) of aortic occlusion produce few irregularities in Dibenamine-treated animals. This point is well illustrated by dividing 20-second periods of occlusion into 5-second units and calculating the percentage of each period occupied by irregularities. Twenty-five such experiments show averages of 2.5, 23, 43 and 47 per cent, respectively, for the four periods. All the differences except the one between the last two periods are statistically significant and indicate that the effect of elevated systemic pressure in sensitizing the myocardium to epinephrine-induced arrhythmias is cumulative.

The effect of elevated arterial pressure may be manifest after release of the aortic occlusion. Irregularities frequently persist for periods up to 10 seconds after the pressure has fallen and in some experiments (fig. 4, left) irregularities

were not observed until immediately after the return of the pressure to preocclusion levels.

Role of Reflex Vagal Activation. The preceding experiments demonstrate the role of systemic arterial pressure in sensitizing the heart to epinephrine-induced cardiac arrhythmias. However, they do not explain the mechanism of this action, which could be either a direct effect of increased left intraventricular pressure or an indirect effect mediated reflexly through the vagi.

A number of workers (9, 10) have stressed the importance of the vagus in epinephrine-induced arrhythmias. It has been suggested that these irregularities represent a ventricular escape phenomenon resulting from simultaneous

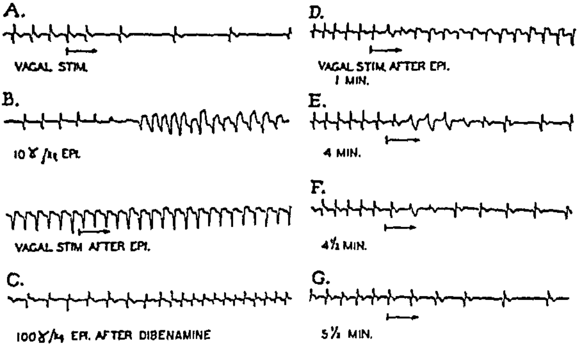


FIG. 5. CARDIAC RESPONSE OF A DOG EQUILIBRATED TO 30 PER CENT CYCLOPROPANE TO VAGAL STIMULATION AS ALTERED BY EPINEPHRINE AND DIBENAMINE

Stimulation begins at arrow and continues to end of each record.

A. Control response.

B. Lack of response in the presence of epinephrine-induced ventricular tachycardia.

C. Cardiac response to epinephrine 30 minutes after treatment with 20 mgm./kgm. of Dibenamine.

D–G. Responses at various intervals after the injection of 100 μgm./kgm. epinephrine in Dibenamine-treated animal. Times indicate interval since epinephrine injection.

direct epinephrine stimulation of the myocardium and reflex vagal inhibition of the sinus node and conducting system. As illustrated in figure 5B stimulation of the peripheral stump of the severed vagus is ineffective in the presence of an epinephrine-induced ventricular tachycardia. However, after Dibenamine protection, the epinephrine-induced sinus tachycardia may be transformed into a ventricular rhythm by strong stimulation of the vagus and this effect gradually disappears as the epinephrine tachycardia subsides (fig. 5, D–G). We have never been able to produce ventricular fibrillation by stimulation of the vagus during the action of injected epinephrine in Dibenamine-treated animals.

In spite of the potential arrhythmia-inducing action of the vagus noted above, several lines of evidence point to the fact that reflex vagal mechanisms are not

important in the production of epinephrine-induced arrhythmias in anesthetized animals (11). Bilateral vagotomy or minimum blocking doses of atropine do not protect against these irregularities (table I). In addition, bilateral vagotomy does not alter the effect of aortic occlusion in inducing irregularities in animals given minimal protective doses of Dibenamine. In 8 animals tested before and after bilateral vagotomy, the average percentage of the 20-second occlusion periods occupied by arrhythmias was 30 per cent and 28 per cent, respectively.

Myocardial Action of Dibenamine. As mentioned above, Dibenamine does not alter the chronotropic or positive inotropic cardiac effects of epinephrine on the mammalian heart. However, it has been observed that large doses of Dibenamine reduce the sinus rate in heart-lung preparations, the maximum rate at which the auricle will follow electrical stimuli (5) and the rate of auricular fibrillation and flutter (12). We have also observed an increase in the threshold

TABLE I

Cardiac responses of dogs equilibrated to 30 per cent cyclopropane–70 per cent oxygen to the intravenous injection of 10 μgm./kgm. epinephrine

TREATMENT	DOSE	NO. ANIMALS	VENTRICULAR FIBRILLATION	AVERAGE DURATION OF	
				All irregularities*	Ventricular tachycardia*
	mgm./kgm.		%		
Control	—	25	32	145 ± 7.2	91 ± 9.6
Dibenamine	20	28	0	1.8 ± 1.0	0
Dibenamine (tested 24-hours after administration)	20	7	0	4.0 ± 1.8	0
2-dibenzylaminoethanol	20	5	40	107 ± 7.1	96 ± 5.0
Atropine sulfate	0.1	8	13	109 ±18	59 ±18
Bilateral vagotomy	—	8	25	131 ±16	76 ±11

* In animals not developing ventricular fibrillation.

for electrically-induced fibrillation (13). These "quinidine-like" effects appear to be best explained on the basis of a slight direct myocardial depressant action of Dibenamine which seems to be completely independent of its adrenergic blocking action. This effect has a duration of only 1 to 2 hours and is produced almost equally by 2-dibenzylaminoethanol which has no adrenergic blocking action.

Several observations indicate that the "quinidine-like" action of Dibenamine is *not* significant in protecting against epinephrine-cyclopropane arrhythmias. Dibenzylaminoethanol is ineffective in preventing epinephrine-induced arrhythmias and a major part of the Dibenamine protection persists for at least 24 hours (table I), as other workers have also observed (14). The protection afforded by Dibenamine against cyclopropane-epinephrine cardiac arrhythmias appears to be correlated with its adrenergic blocking action, but the precise mechanism by which its protective action on the myocardium is exerted is still obscure.

Discussion. The results reported indicate that at least two factors are involved in the protection afforded by Dibenamine against epinephrine-cyclopropane cardiac arrhythmias. Some direct action of Dibenamine on the myocardium is involved inasmuch as intermediate doses of the drug reverse the pressor action of epinephrine without protecting against arrhythmias, while larger doses have both effects. Garb and Chenoweth (15), studying cat hearts sensitized by volatile hydrocarbons, also demonstrated a dissociation of these two actions of Dibenamine. Although differing from ours in certain details, their results emphasize the fact that reversal of the pressor action of epinephrine and protection against epinephrine-induced cardiac arrhythmias are not necessarily causally related. At present no definitive explanation of the mechanism of direct myocardial protection afforded by Dibenamine can be given. It may be that an adequate answer to this question will not be forthcoming until the mechanism by which epinephrine causes the development of ectopic foci of impulse formation in a sensitized myocardium is more fully understood.

Although Dibenamine does not protect against cardiac arrhythmias simply by reversing the pressor response to epinephrine, systemic arterial pressure is a factor sensitizing the heart to epinephrine-induced arrhythmias. Our results indicate that absolute pressure rather than increase in pressure is the important factor in sensitizing the heart to ectopic rhythms. The fact that the heart is more sensitive to epinephrine-induced irregularities when it is working against a high systemic pressure was also observed by Moe and coworkers (16) and may explain their conclusions that the rise in systemic arterial pressure caused by epinephrine was essential to the production of arrhythmias and that Dibenamine protected against arrhythmias simply because it reversed this pressor action.

The mechanism by which systemic hypertension predisposes to cardiac arrhythmias is not clear. The elevated pressure does not act to any major degree through a reflex vagal mechanism. It may act to produce a local relative myocardial hypoxia by increasing the work required of the left ventricle. Hypoxia is known to predispose to irregularities in the presence of cyclopropane (17, 18), and the electrocardiographic changes (depressed S-T segment) seen in a few experiments in which elevated pressure did not produce frank arrhythmias are compatible with some degree of myocardial hypoxia.

In our experiments, the maximum tolerated generalized hypoxia allowed the production of only minor epinephrine-cyclopropane cardiac irregularities in Dibenamine-treated animals. However, this does not preclude consideration of a relative myocardial hypoxia as an etiological factor in the initiation of irregularities by increased systemic pressure. Under the strain of extra work the myocardial hypoxia may be quite unevenly distributed, and the resultant uneven alterations in myocardial conduction and excitability might be expected to be effective in inducing arrhythmias.

A second possibility is that increased pressure sensitizes the heart by producing some degree of cardiac dilatation, in agreement with the suggestion of Bouckaert and Heymans (19) that cardiac dilatation is a prerequisite to ventricular fibrilla-

tion. Either of these mechanisms of sensitization would offer an explanation for the cumulative effect of systemic hypertension.

The fact that the cyclopropane-sensitized heart is most prone to develop arrhythmias shortly after the injected epinephrine first reaches it rather than at the peak of epinephrine concentration points to some type of accommodation, but the mechanism involved is obscure. The development of a sinus tachycardia does not appear to be a major factor inasmuch as an increase in control heart rate induced by atropine does not afford protection (table I). It may be that epinephrine-induced changes in coronary circulation or a decreased tendency to cardiac dilatation due to the inotropic action of epinephrine are involved.

SUMMARY

Experiments on dogs under cyclopropane anesthesia indicate that the protection against epinephrine-induced cardiac arrhythmias afforded by Dibenamine and related adrenergic blocking agents is due to two factors:

1. A direct cardiac action which requires larger doses of blocking agent than are necessary to reverse completely the peripheral pressor effect of the injected epinephrine. This protection is largely independent of a transient "quinidine-like" action on the myocardium shared by related compounds devoid of adrenergic blocking activity.

2. Prevention of an elevated blood pressure during the period of epinephrine action. Artificial elevation of the blood pressure by occlusion of the thoracic aorta has been shown to act as an added stress capable of producing irregularities in many hearts otherwise protected by Dibenamine. The effects of aortic occlusion were found to be dependent upon the final level of systemic arterial pressure attained and to be largely independent of the magnitude of the rise in pressure. Sustained pressure elevation produces cumulative effects.

The heart was found to be most prone to develop arrhythmias shortly after the injected epinephrine first reaches it rather than at the time of maximum epinephrine concentration in the blood stream.

The influence of systemic arterial pressure on the production of cardiac arrhythmias does not appear to be mediated through the vagi.

The development of a relative myocardial hypoxia and the production of some degree of cardiac dilatation are discussed as possible mechanisms by which systemic hypertension might sensitize the myocardium to epinephrine-induced arrhythmias.

REFERENCES

1. NICKERSON, M., AND GOODMAN, L. S.: Proc. Am. Fed. Clin. Res., 2: 109, 1945.
2. NICKERSON, M., AND GOODMAN, L. S.: THIS JOURNAL, 89: 167, 1947.
3. NICKERSON, M., SMITH, S. M., AND GOODMAN, L. S.: Fed. Proc., 5: 195, 1946.
4. NICKERSON, M., AND SMITH, S. M.: Anesthesiology (submitted).
5. ACHESON, G. H., FARAH, H., AND FRENCH, G. N.: Fed. Proc., 6: 305, 1947.
6. STUTZMAN, J. W., AND ALLEN, C. R.: Proc. Soc. Exper. Biol. & Med., 47: 218, 1941.
7. WOODBURY, L. A., NICKERSON, M., AND WOODBURY, J. W.: to be published.
8. NICKERSON, M., BROWN, H. O., AND SMITH, S. M.: Am. J. Med., in press.
9. PETZETAKIS, M., AND VLACHLIS, G.: Arch. des mal. du Coeur., 23: 333, 1930.

10. Wilburne, M., Surtshin, A., Rodbard, S., and Katz, L. N.: Am. Heart J., 34: 860, 1947.
11. Nickerson, M., Toman, J. E. P., and Hecht, H. H.: Fed. Proc., 6: 361, 1947.
12. Acheson, G. H., and Farah, H.: personal communication.
13. Nickerson, M., and Bullock, F. S.: unpublished results.
14. Wilburne, M., Katz, L. N., Rodbard, S., and Surtshin, A.: This Journal, 90: 215, 1947.
15. Garb, S., and Chenoweth, M. B.: Fed. Proc., 7: 220, 1948.
16. Moe, G. K., Malton, S. D., Freyburger, W., and Rennick, B.: J. Lab. & Clin. Med., 32: 1415, 1947.
17. Seevers, M. H., Meek, W. J., Rovenstine, E. A., and Stiles, J. A.: This Journal, 51: 1, 1934.
18. Robbins, B. H., and Baxter, J. H.: This Journal, 61: 162, 1937.
19. Bouckaert, J. J., and Heymans, C.: Compt. rend. Soc. de Biol., 105: 878, 1941.

THE DISTRIBUTION IN RABBIT TISSUES OF INTRAVENOUSLY INJECTED IODINE AS SHOWN BY THE RADIOISOTOPE, I 130

WALTER MANN, WILLIAM F. BALE, HAROLD C. HODGE, AND STAFFORD L. WARREN

Department of Radiology and Division of Pharmacology and Toxicology, School of Medicine and Dentistry, University of Rochester, Rochester, New York

Received for publication September 15, 1948

Reports recently have been published by Hertz and Roberts (1) and by Chapman and Evans (2) dealing with the treatment of hyperthyroidism by means of internal irradiation. Radioactive iodine, orally administered, is the therapeutic agent, and at present, these authors believe that it is at least an alternate medical method for the control of thyrotoxicosis. Lately, also, a case of metastatic thyroid cancer has been treated with radioiodine by Marinelli and coworkers (3). It is, therefore, important to study the distribution of radioactive iodine in various organs; and, since one isotope (I 130) has a relatively short half-life, the period immediately after administration has special interest.

In this study, radioactive iodine determinations were carried out on the various tissues of animals in the period from ½ to 12 hours after intravenous radioiodine administration; during this time, if the 12.6 hour isotope is used in irradiation work, the radiation rate is at its highest and a large fraction of the dose is delivered. Some radioisotope data of this kind have already been published (4); in addition, in the current work the concentration of total iodine in the tissues has been determined by microchemical analyses.

The fraction of an administered dose of radioactive iodine found in tissues other than the thyroid gland is relatively low, thus minimizing the possibility of radiation-induced pathology in these tissues. Unfortunately, data were not collected on the radioiodine content of bone marrow, lymph nodes, and ovaries; this deficiency should be corrected in light of the known radiation sensitivity of these tissues. In the absence of definitive data, however, it may be assumed that the radioiodine concentration was of the same order as that in the blood, liver, lung, kidney, spleen, muscle, and heart.

Methods. The radioactive iodine was prepared by proton bombardment of tellurium in the cyclotron of the University of Rochester Physics Department. The iodine was separated from the tellurium and prepared for intravenous injection in the form of iodide as previously described (3). An amount of carrier iodine was added such that each animal received a single dose of 15 micrograms of iodine per kgm. of body weight; this small amount should not greatly alter the normal metabolic distribution of iodine. The weight of the radioactive iodine (I 130) was negligible. Each rabbit received 4 to 9 microcuries per kgm. body weight of radioiodine.

Normal, young adult, New Zealand, white rabbits were used throughout these experiments. These rabbits were all from the same stock, weighing 2.5 to 3.0 kgm., and were approximately of the same age (5–6 months). They were housed separately in wire-bottomed cages and given a diet of oats and dry alfalfa for 2 to 3 weeks before use; this diet was available after the injection of radioactive iodine. Tap water was provided at all times.

The city water contains approximately 5 parts of iodine per billion, and no iodine was added to it

At the time of sacrifice, the various organs were dissected out, weighed and samples taken for analysis Blood was withdrawn by heart puncture just before sacrifice The radioactivity determinations and the chemical iodine determinations were performed as described previously (5)

TABLE I

The changes in distribution with time after administration of radioiodine together with the total iodine content of thyroid and blood

RABBIT SERIAL NO	TIME	THYROID					BLOOD	
		Wt	I_{131} dose per gram (×100)	I_{131} dose per whole gland	I conc	I (γ)/gland	I_{131} dose per gram (×100)	I conc
	hours	*grams*	%	%	*γ/100 grams*		%	*γ/100 grams*
28	0 5	0 22	372	0 8	59,000	126 8	7 5	—
30	0 5	0 20	4620	9 1	17,980	35 4	7 4	36 6
31	0 5	0 17	617	1 0	24,100	40 9	9 1	32 9
10	0 66	0 25	318	0 8	24,050	60 2	—	—
2	1 0	0 53	1850	6 1	6,590	21 8	4 6	—
3	1 0	0 22	2120	4 6	10,560	23 2	5 9	3 0
4	1 0	0 11	1500	1 7	102,100	112 2	—	—
5	1 0	0 16	910	1 5	102,000	162 0	5 5	2 8
6	1 0	0 15	1900	2 8	10,070	16 0	6 1	3 1
7	1 0	0 22	1070	4 9	56,400	124 8	6 5	5 7
8	1 0	0 14	1400	2 0	5,630	7 9	7 8	6 0
9	1 0	0 65	654	4 2	17,000	111 0	5 7	6 3
11	2 0	0 23	2490	5 7	11,790	27 5	—	—
14	2 0	0 30	2820	8 4	8,900	26 7	4 9	7 3
15	2 0	0 18	4420	8 0	6,020	10 9	7 1	3 7
16	2 0	0 44	10,490	46 0	5,160	22 7	1 8	7 6
17	2 0	0 25	6370	16 0	9,050	22 6	5 2	3 0
18	2 0	0 32	2380	7 6	18,050	57 7	3 7	2 6
19	2 0	0 24	6280	15 0	8,380	20 1	4 6	2 3
20	6 0	0 16	7150	11 0	24,600	39 3	3 8	5 3
21	6 0	0 16	8390	13 0	18,050	28 9	5 5	6 1
22	6 0	0 22	3270	7 2	111 200	245 0	4 2	21 0
23	6 0	0 38	10,700	41 0	13,400	51 1	2 8	24 9
24	12 0	0 22	4880	10 0	49,900	107 2	1 3	39 2
25	12 0	0 21	16,080	34 0	27,100	56 6	3 8	41 3
26	12 0	0 11	16,180	18 0	45,200	49 7	4 9	44 1
27	12 0	0 12	26,800	31 0	22,200	25 8	2 2	42 6

DATA AND DISCUSSION Tables I and II show the distribution of radioactive iodine in the tissues at various times after injection, the I 127 (total iodine) concentrations are also given The amounts of radioactive iodine per hundred grams of tissue are expressed as percentages of the dose injected All radioactivity values were corrected to zero time For the thyroid gland, the results are also calculated as a percentage of the dose in the entire gland The iodine concentrations determined chemically are in micrograms per hundred grams, for the thyroid gland, the total amount present is also listed

Thyroid. As is seen from the tables and figures, the thyroid gland builds up a concentration of radioiodine which is, at the earliest time studied (½ hour after injection), at least one hundred times greater than that in other tissues. The concentration in the thyroid continues to rise during the twelve-hour period observed in these experiments. At this time (12 hours) the radioactive iodine con-

TABLE II

The changes in distribution with time after administration of radioiodine together with the total iodine content of selected tissues

RABBIT SERIAL NO.	MUSCLE		KIDNEY		LUNG		LIVER		SPLEEN		HEART	
	I_{131} dose per gram (×100)	I conc.	I_{131} dose per gram (×100)	I conc.	I_{131} dose per gram (×100)	I conc.	I_{131} dose per gram (×100)	I conc.	I_{131} dose per gram (×100)	I conc.	I_{131} dose per gram (×100)	I conc.
	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams
28	1.4	43.5	5.5	31.1	5.1	63.6	1.7	40.8	4.9	180.8	4.3	61.9
30	1.3	37.6	7.1	41.8	5.7	53.2	1.9	42.1	2.0	84.4	4.3	71.5
31	2.3	41.6	—	—	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	—	—	—
2	4.6	—	10.6	3.8	3.0	2.9	1.7	—	2.6	—	3.5	2.4
3	1.6	1.1	7.8	7.5	3.7	3.7	2.3	1.3	4.4	41.6	3.1	3.5
4	—	—	17.6	39.4	—	—	—	—	—	—	—	—
5	—	—	7.4	1.9	—	—	—	—	—	—	—	—
6	1.1	1.2	11.4	1.9	3.6	6.5	2.3	2.9	14.7	39.2	2.8	2.0
7	0.8	5.3	9.7	8.1	7.4	6.5	2.7	11.4	3.0	92.1	3.2	1.7
8	—	—	—	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	1.6	1.5	3.9	—	—	—
14	0.7	0.52	7.6	12.3	8.8	5.3	2.1	2.4	2.7	—	3.0	3.1
15	3.1	1.2	—	—	—	—	—	—	—	—	—	—
16	0.3	0.59	4.2	6.4	1.6	4.6	0.8	2.0	0.9	—	2.1	1.7
17	0.9	0.84	—	—	—	—	—	—	4.1	—	—	—
18	0.4	—	2.6	1.6	1.0	—	1.8	2.4	9.2	1.7	2.4	3.1
19	—	—	—	—	—	—	—	—	—	—	—	—
20	0.5	—	7.2	11.4	2.7	4.6	1.2	5.8	—	—	2.9	5.9
21	0.8	1.2	—	—	—	—	—	—	—	—	—	—
22	0.5	13.2	4.4	17.7	3.6	20.1	1.7	19.1	—	—	3.0	35.4
23	0.6	18.5	—	—	—	—	—	—	2.1	38.0	—	—
24	0.3	42.2	4.1	58.7	1.0	80.1	0.5	41.6	8.2	164.4	0.9	56.6
25	0.5	—	—	—	—	—	—	—	—	—	—	—
26	0.6	51.0	7.2	11.7	4.1	69.6	1.3	37.8	2.5	160.0	2.4	49.2
27	0.3	37.5	—	—	—	—	—	—	—	—	—	—

centration is more than one thousand times that in other tissues (figures 1, 2 and 3).

Although over a fifth of the total dose (15 micrograms per kgm. body weight) was found in the thyroid glands at twelve hours, this fraction is lower than the amount found by Perlman, Chaikoff and Morton (14) when tracer doses were given to rats. With such doses, almost 60 per cent of the dose had appeared in

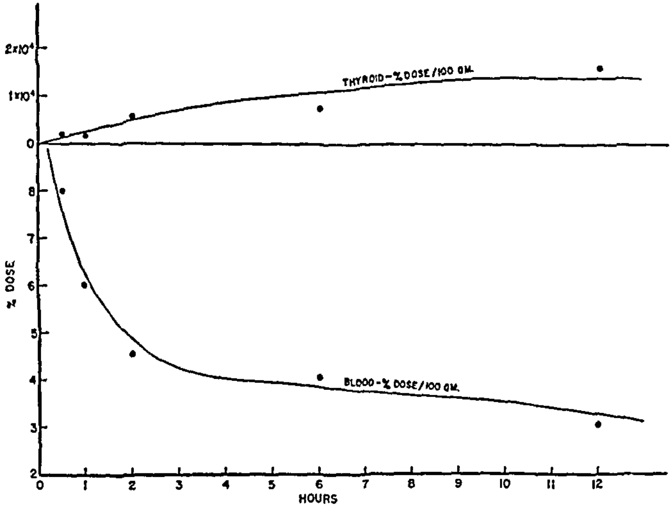


FIG. 1. CHANGES DURING THE INITIAL 12 HOURS IN RADIOIODINE CONTENT OF THYROID GLAND AND BLOOD

In the thyroid, the highest value occurs at the 12-hour interval. The blood contains nearly one-third as much of the original dose at 12 hours as it contained at one-half hour.

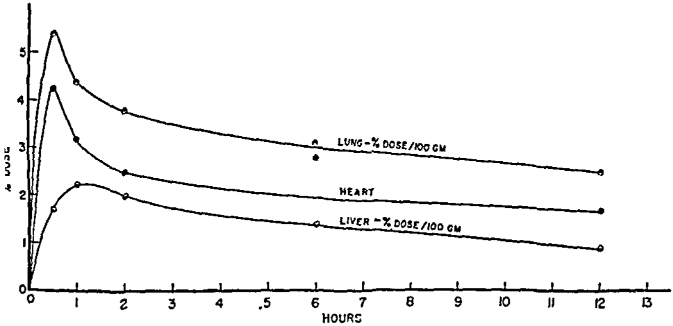


FIG. 2. CHANGES DURING THE INITIAL 12 HOURS IN RADIOIODINE CONTENT OF LUNG, HEART AND LIVER

After a period of rapid increase in radioiodine content during the half-hour, a roughly parallel course of slowly decreasing content is described for all three tissues. After 2 hours, the amounts present are somewhat less than those in the blood (fig. 1) but are of the same order of magnitude.

Thyroid. As is seen from the tables and figures, the thyroid gland builds up a concentration of radioiodine which is, at the earliest time studied (½ hour after injection), at least one hundred times greater than that in other tissues. The concentration in the thyroid continues to rise during the twelve-hour period observed in these experiments. At this time (12 hours) the radioactive iodine concentration is more than one thousand times that in other tissues (figures 1, 2 and 3).

TABLE II

The changes in distribution with time after administration of radioiodine together with the total iodine content of selected tissues

RABBIT SERIAL NO.	MUSCLE		KIDNEY		LUNG		LIVER		SPLEEN		HEART	
	I_{130} dose per gram (×100)	I conc.	I_{130} dose per gram (×100)	I conc.	I_{130} dose per gram (×100)	I conc.	I_{130} dose per gram (×100)	I conc.	I_{130} dose per gram (×100)	I conc.	I_{130} dose per gram (×100)	I conc.
	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams	%	γ/10 grams
28	1.4	43.5	5.5	31.1	5.1	63.6	1.7	40.8	4.9	180.8	4.3	61.9
30	1.3	37.6	7.1	41.8	5.7	53.2	1.9	42.1	2.0	84.4	4.3	71.5
31	2.3	41.6	—	—	—	—	—	—	—	—	—	—
10	—	—	—	—	—	—	—	—	—	—	—	—
2	4.6	—	10.6	3.8	3.0	2.9	1.7	—	2.6	—	3.5	2.4
3	1.6	1.1	7.8	7.5	3.7	3.7	2.3	1.3	4.4	41.6	3.1	3.5
4	—	—	17.6	39.4	—	—	—	—	—	—	—	—
5	—	—	7.4	1.9	—	—	—	—	—	—	—	—
6	1.1	1.2	11.4	1.9	3.6	6.5	2.3	2.9	14.7	39.2	2.8	2.0
7	0.8	5.3	9.7	8.1	7.4	6.5	2.7	11.4	3.0	92.1	3.2	1.7
8	—	—	—	—	—	—	—	—	—	—	—	—
9	—	—	—	—	—	—	—	—	—	—	—	—
11	—	—	—	—	—	—	1.6	1.5	3.9	—	—	—
14	0.7	0.52	7.6	12.3	8.8	5.3	2.1	2.4	2.7	—	3.0	3.1
15	3.1	1.2	—	—	—	—	—	—	—	—	—	—
16	0.3	0.59	4.2	6.4	1.6	4.6	0.8	2.0	0.9	—	2.1	1.7
17	0.9	0.84	—	—	—	—	—	—	4.1	—	—	—
18	0.4	—	2.6	1.6	1.0	—	1.8	2.4	9.2	1.7	2.4	3.1
19	—	—	—	—	—	—	—	—	—	—	—	—
20	0.5	—	7.2	11.4	2.7	4.6	1.2	5.8	—	—	2.9	5.9
21	0.8	1.2	—	—	—	—	—	—	—	—	—	—
22	0.5	13.2	4.4	17.7	3.6	20.1	1.7	19.1	—	—	3.0	35.4
23	0.6	18.5	—	—	—	—	—	—	2.1	38.0	—	—
24	0.3	42.2	4.1	58.7	1.0	80.1	0.5	41.6	8.2	164.4	0.9	56.6
25	0.5	—	—	—	—	—	—	—	—	—	—	—
26	0.6	51.0	7.2	11.7	4.1	69.6	1.3	37.8	2.5	160.0	2.4	49.2
27	0.3	37.5	—	—	—	—	—	—	—	—	—	—

Although over a fifth of the total dose (15 micrograms per kgm. body weight) was found in the thyroid glands at twelve hours, this fraction is lower than the amount found by Perlman, Chaikoff and Morton (14) when tracer doses were given to rats. With such doses, almost 60 per cent of the dose had appeared in

mined iodine concentrations vary considerably among the tissues as had been noted by previous workers (8) in studies not involving the use of radioiodine; consequently, no readily apparent relationship between the chemically-determined iodine and the radioiodine content of the tissues is displayed, except the obvious one that the thyroid with its high content of iodine picks up a relatively high amount of radioiodine.

The distribution of radioiodine in the tissues is quite similar to the percentages found by Perlman, Chaikoff and Morton. This is interesting because they administered tracer doses of radioiodine to rabbits subcutaneously; whereas in the experiments reported here, 15 micrograms of iodine per kgm. were injected intravenously. The *blood* value at 5 hours in their experiments was 5.6 per cent of the dose per 100 grams; in these experiments at 6 hours it is 4 per cent of the dose per 100 grams. At the same time periods, the *kidney* values are 8.1 per cent and 5.8 per cent, respectively; these are certainly of the same order of magnitude.

Insofar as higher thyroid collections have been reported in hyperthyroid humans (7) than in animals (4), even lower values of radioiodine in tissues other than the thyroid might be found in hyperthyroid humans. Therefore, one might predict that the possibility of radiation-induced pathology should be slight in these tissues. Nevertheless, special attention should be given to such organs as the kidneys, since a large proportion of a single dose is excreted via the urine in a few days, and the possibility of a delayed, radiation-induced malignancy appearing in internally-irradiated organisms must be kept in mind (9). In these tests the total dose of radiation was so small that the radioactivity in the kidney almost certainly never reached dangerous levels.

SUMMARY

The distribution of intravenously-administered radioiodine in the tissues of rabbits, up to 12 hours after injection, has been measured; the total iodine present in these tissues has also been determined chemically. The thyroid gland contained relatively high percentages of the injected dose; the other tissues studied, although variations are observed from tissue to tissue, all have a relatively low concentration of radioiodine.

From this work and the data of others, it is concluded that to produce maximal concentrations of radioiodine in the thyroid gland, the total dose of iodine (I 127) should be kept as small as possible.

REFERENCES

1. Hertz, S., and Roberts, A. J. Clin. Investigation, 21: 624, 1942.
2. Chapman, E. M., and Evans, R. D. J. A. M. A., 131: 86, 1946.
3. Seidlin, S. M., Marinelli, L. D., and Oshry, E. J. A. M. A., 132: 838, 1946.
4. Perlman, I., Chaikoff, I. L., and Morton, M. E. J. Biol. Chem., 139: 433, 1941.
5. Mann, W., Leblond, C. P., and Warren, S. L. J. Biol. Chem., 142: 905, 1942.
6. Hertz, S., Roberts, A., and Evans, R. D. Proc. Soc. Exp. Biol. and Med., 38: 510, 1938.
7. Hertz, S., Roberts, A., and Salter, W. T. J. Clin. Investigation, 21: 25, 1942.
8. Maurer, E., and Ducrue, H. Biochem. Zeitschr., 193: 356, 1928.
9. Mitchell, J. S. Brit. J. Cancer, 1: 1, 1947.

STUDIES ON DIETHYLAMINOETHANOL[1]

I. Physiological Disposition and Action on Cardiac Arrhythmias

BENJAMIN ROSENBERG, HERBERT J. KAYDEN, PHILIP A. LIEF, LESTER C. MARK, J. MURRAY STEELE, and BERNARD B. BRODIE

Medical and Research Services, Third (New York University) Medical Division, Goldwater Memorial Hospital, and Departments of Medicine, Anesthesia and Biochemistry, New York University College of Medicine, New York, N. Y.

Received for publication September 15, 1948

Procaine has been shown to protect dogs during cyclopropane anesthesia from the development of ventricular tachycardia and ventricular fibrillation induced by the injection of epinephrine (1, 2). Subsequently anesthesiologists have employed intravenous procaine to prevent and abolish ventricular arrhythmias in the anesthetized human (3). Recent work in this laboratory (4) has shown that procaine administered intravenously in man is hydrolyzed in the body with unusual rapidity to p-aminobenzoic acid and diethylaminoethanol. The high concentration and relative persistence of diethylaminoethanol in the plasma suggested the possibility that this compound might account for some or all of the pharmacologic properties of the parent drug, such as the quinidine-like action on the heart, local anesthesia, analgesia, spasmolytic action and anti-allergic action.

This report is divided into two sections. The first is concerned with the physiological disposition of diethylaminoethanol in the body. It describes those factors which affect the plasma level of the drug and therefore presumably the therapeutic effect. The second section describes the quinidine-like action of the drug on the heart of the dog and man.

SECTION I. THE PHYSIOLOGICAL DISPOSITION OF DIETHYLAMINOETHANOL

Methods. Chemical method: The diethylaminoethanol determinations were made by means of a method recently devised in this laboratory (4). This method has been shown to be specific in that the measurement includes no transformation products of diethylaminoethanol.

Preparation of tissues: Small pieces of tissues were removed from freshly sacrificed animals. The samples were weighed and then homogenized in a motor driven glass device which fragmented most cells (5).

Experimental data. *Excretion and transformation of diethylaminoethanol.* (1) Intravenous administration: Two subjects received 5.6 grams of diethylaminoethanol hydrochloride[2] intravenously in 11.2 per cent solution. Urine col-

[1] This study was supported by grants from E. R. Squibb and Sons, New Brunswick, N. J., and the Institute for the Study of Analgesic and Sedative Drugs.

[2] The diethylaminoethanol used in these experiments was obtained through the courtesy, of the Department of Medical Research, Winthrop-Stearns, Inc., New York, N. Y., Novocol Chemical Mfg. Co., Inc., Brooklyn, N. Y., and E. R. Squibb and Sons, New Brunswick, N. J. Dr. D. J. Graubard kindly made available to us additional diethylaminoethanol (Winthrop-Stearns).

lections were made over a period of 48 hours. Plasma levels of the compound were measured at various intervals.

Subject H excreted 21 and subject P 20 per cent of the administered dose as unchanged diethylaminoethanol. The rest of the drug was transformed in the body. The nature of the transformation has not been ascertained. The plasma

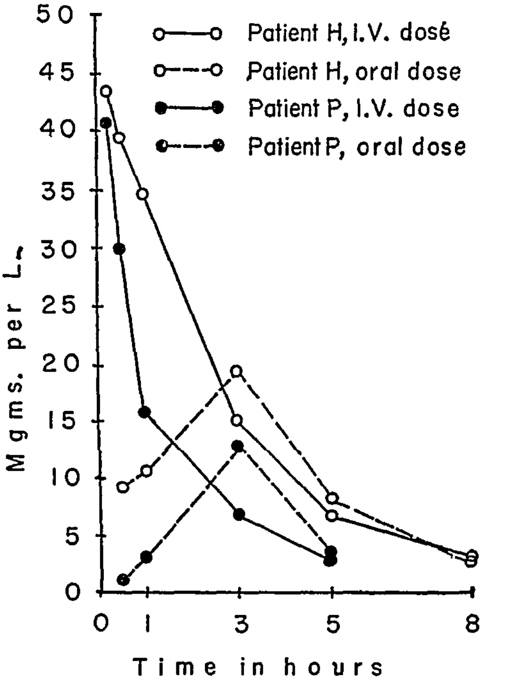


FIG. 1. A COMPARISON OF PLASMA LEVELS OF DIETHYLAMINOETHANOL AFTER INTRAVENOUS AND ORAL ADMINISTRATION OF 5.6 GRAMS OF THE HYDROCHLORIDE

levels of the drug declined rapidly, and at the end of 8 hours were almost zero, indicating that the drug was almost completely metabolized (fig. 1).

(2) Oral administration: The same 2 subjects received 5.6 grams of diethylaminoethanol hydrochloride orally in aqueous solution. The urine was again collected over a period of 48 hours and plasma levels were measured at various intervals.

The amount of unchanged drug excreted in the urine by subject H was 27, and

by subject P, 23 per cent. The rough similarity of the excretion of the drug after its oral administration with that after its intravenous administration suggests that the absorption of diethylaminoethanol from the gastrointestinal tract is essentially complete. Peak plasma levels were achieved in about 3 hours (fig. 1). The peak levels for the oral route are much lower than the levels which occur with the intravenous route.

The distribution of diethylaminoethanol in various tissues. A dog received by intravenous infusion 11 grams of diethylaminoethanol hydrochloride over a one-hour period. Three hours later a blood sample was drawn and the animal was sacrificed. Diethylaminoethanol in most organ tissues was considerably higher than in plasma, indicating extensive localization of the drug (table I).

TABLE I

Distribution of diethylaminoethanol in the dog

The distribution of diethylaminoethanol was examined in various dog tissues. The studies were made 3 hours after the intravenous infusion of 11 grams of the hydrochloride drug. The dog weighed 15 4 kgm.

TISSUE	CONCENTRATION OF DIETHYLAMINOETHANOL
	mgm /kgm
Plasma	70
Muscle	72
Heart	134
Brain	223
Lung	447
Liver	995
Spleen	1227
Cerebrospinal fluid	37

The degree of binding on the non-diffusible constituents of plasma was deter mined by dialysis against isotonic phosphate buffer of pH 7.4 at 37°C. for 18 hours. Visking cellulose sausage casings were utilized as dialysis bags. Little or none of the diethylaminoethanol was found to be bound. This is surprising in view of its localization in tissues.

SECTION II. ACTION OF DIETHYLAMINOETHANOL ON CARDIAC ARRHYTHMIAS

The protective action of diethylaminoethanol against cyclopropane-epinephrine-induced cardiac arrhythmias in dogs. The technique described by Meek (2) was used to produce cardiac arrhythmias in dogs. The animals were maintained at second plane anesthesia with cyclopropane for thirty minutes. Preanesthetic medication consisted of morphine sulfate, 1 mgm. per kgm., and atropine sulfate, 0.04 mgm. per kgm., injected subcutaneously one hour before each experiment. A test dose of epinephrine sufficient to produce ventricular premature contractions followed by ventricular tachycardia (0.01–0.015 mgm. per kgm. in 5 cc. of normal saline) was injected intravenously at the rate of 1 cc. per 10 seconds. After the cardiac response to the test dose of epinephrine had worn off, a second

dose of the drug invariably produced arrhythmias again Electrocardiograms (lead II) were taken continuously during the experiments

The cardiac response of anesthetized dogs to the test dose of epinephrine was compared before and after the intravenous administration of diethylaminoethanol In this way each animal acted as its own control After the effect of the test dose of epinephrine was dissipated diethylaminoethanol hydrochloride was administered intravenously by a single quick injection in doses of 2 to 7 grams in 11 2 per cent aqueous solution Three minutes later, the effect of another test dose of epinephrine was observed

TABLE II

Protective action of diethylaminoethanol against epinephrine induced cardiac arrhythmias in dogs

A test dose of epinephrine (0 01–0 015 mgm /kgm) sufficient to produce ventricular premature contractions and ventricular tachycardia was injected intravenously into dogs under cyclopropane anesthesia After the effect of the drug had subsided diethylamino ethanol was injected Three minutes later the test dose of epinephrine was repeated and its effect noted

EXPT NO	WT OF DOG	DOSE OF DIETHYLAMINO ETHANOL	DEGREE OF PROTECTION AGAINST EPINEPHRINE*
	kgm	*grams*	
1	15	2	None
2	8 5	2	None
3	8 8	2	Complete
4	12	3	Complete
5	13	4	Partial
6	12	5	Complete
7	13 5	7	Complete

* Protection is considered complete when no ventricular arrhythmia results, it is considered partial when ventricular premature contractions occur without ventricular tachycardia

Diethylaminoethanol gave complete protection to 4 dogs against the development of ventricular premature contractions and ventricular tachycardia (table II, expts 3, 4, 6 and 7) In figure 2 are shown excerpts from the ECG tracings of a typical experiment (expt 4) The duration of the protective action was not studied since it was not the purpose of these experiments to quantify the effect of the diethylaminoethanol, but merely to indicate its qualitative action in dogs prior to human trial Two animals, however, (expts 3 and 7) were protected against the effect of further doses of epinephrine 15 minutes after the diethylaminoethanol administration In one experiment (expt 5) only partial protection was afforded by diethylaminoethanol in that ventricular premature contractions but not a ventricular tachycardia developed after the administration of epinephrine Two animals (expts 1 and 2) which received only 2 grams each of diethylaminoethanol were not protected from ventricular tachycardia

p-Aminobenzoic acid, the other product of the hydrolysis of procaine *in vivo*,

by subject P, 23 per cent. The rough similarity of the excretion of the drug after its oral administration with that after its intravenous administration suggests that the absorption of diethylaminoethanol from the gastrointestinal tract is essentially complete. Peak plasma levels were achieved in about 3 hours (fig. 1). The peak levels for the oral route are much lower than the levels which occur with the intravenous route.

The distribution of diethylaminoethanol in various tissues. A dog received by intravenous infusion 11 grams of diethylaminoethanol hydrochloride over a one-hour period. Three hours later a blood sample was drawn and the animal was sacrificed. Diethylaminoethanol in most organ tissues was considerably higher than in plasma, indicating extensive localization of the drug (table I).

TABLE I

Distribution of diethylaminoethanol in the dog

The distribution of diethylaminoethanol was examined in various dog tissues. The studies were made 3 hours after the intravenous infusion of 11 grams of the hydrochloride drug. The dog weighed 15 4 kgm.

TISSUE	CONCENTRATION OF DIETHYLAMINO-ETHANOL
	mgm /kgm
Plasma	70
Muscle	72
Heart	134
Brain	223
Lung	447
Liver	995
Spleen	1227
Cerebrospinal fluid	37

The degree of binding on the non-diffusible constituents of plasma was deter mined by dialysis against isotonic phosphate buffer of pH 7.4 at 37°C. for 18 hours. Visking cellulose sausage casings were utilized as dialysis bags. Little or none of the diethylaminoethanol was found to be bound. This is surprising in view of its localization in tissues.

SECTION II. ACTION OF DIETHYLAMINOETHANOL ON CARDIAC ARRHYTHMIAS

The protective action of diethylaminoethanol against cyclopropane-epinephrine-induced cardiac arrhythmias in dogs. The technique described by Meek (2) was used to produce cardiac arrhythmias in dogs. The animals were maintained at second plane anesthesia with cyclopropane for thirty minutes. Preanesthetic medication consisted of morphine sulfate, 1 mgm. per kgm., and atropine sulfate, 0.04 mgm. per kgm., injected subcutaneously one hour before each experiment. A test dose of epinephrine sufficient to produce ventricular premature contractions followed by ventricular tachycardia (0.01–0.015 mgm. per kgm. in 5 cc. of normal saline) was injected intravenously at the rate of 1 cc. per 10 seconds. After the cardiac response to the test dose of epinephrine had worn off, a second

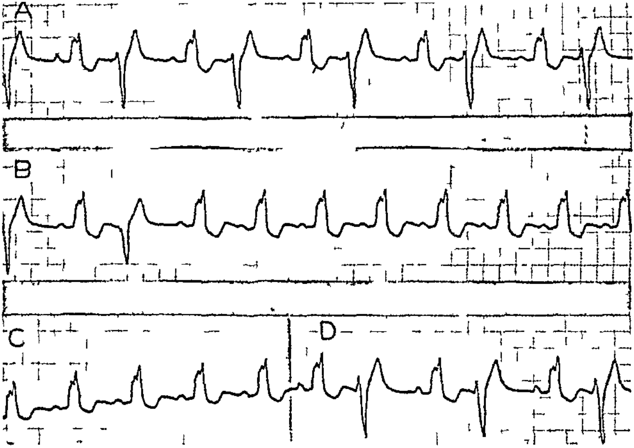


Fig 3 The Effect of Diethylaminoethanol on Coupled Extrasystolic Rhythm in Man

Records are excerpts (retouched) from ECG tracings (lead II) (A) Control tracing exhibiting coupling (B) Reversion to normal rhythm immediately following the intravenous injection of 0 5 gram of diethylaminoethanol (C) 3 minutes later (D) 9 minutes later, return of coupling

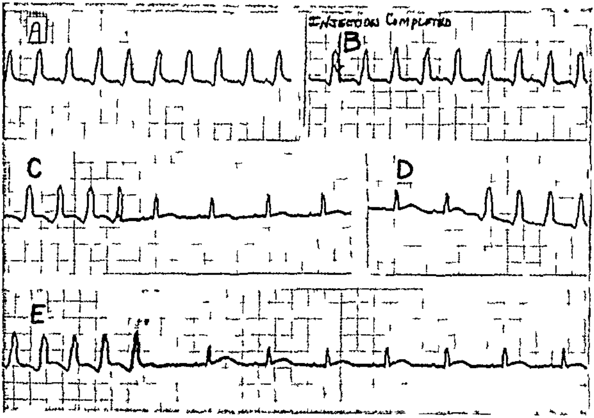


Fig 4 The Effect of Diethylaminoethanol on Ventricular Tachycardia in Man (case 2)

Records are excerpts (retouched) from ECG Tracings (lead I) (A) Control tracing Ventricular tachycardia (B) Tracing immediately following the intravenous injection of 2 grams of diethylaminoethanol (C) Two minutes later showing reversion to normal sinus rhythm (D) Return to ventricular tachycardia 25 minutes later (E) Tracing recorded just at the completion of a further injection of 2 5 grams of diethylaminoethanol, showing reversion to normal sinus rhythm

of diethylaminoethanol hydrochloride ranged from 0.5 to 5 grams in 11.2 per cent aqueous solution injected at a rate of 1 gram per minute.

The ectopic ventricular beats disappeared in 13 of the 14 cases (see example, fig. 3). The effect of diethylaminoethanol was usually apparent within 5 minutes after the start of injection. The duration of effect was not predictable. In 10 cases it was transient, lasting from 3 to 20 minutes. In 3 cases the ectopic rhythm had not reappeared within a week. The one patient who failed to respond had rheumatic heart disease and had been digitalized to toxicity.

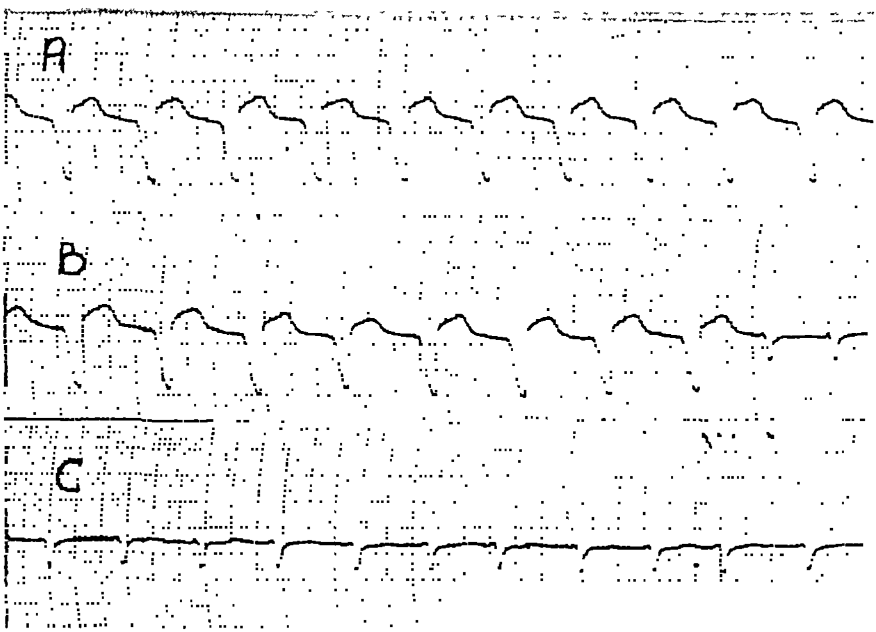


FIG. 5. EFFECT OF DIETHYLAMINOETHANOL ON VENTRICULAR TACHYCARDIA IN MAN (CASE 3)

Records are excerpts from ECG tracings (lead II). (A) Control tracing. Ventricular tachycardia. (B) Tracing recorded just after the intravenous administration of 5 grams of diethylaminoethanol. The reversion to the subject's basic rhythm of auricular fibrillation is shown in the last two complexes. (C) Tracing taken 24 hours later. Ventricular tachycardia has not returned.

Since ventricular tachycardia is a procession of ventricular premature contractions, the effect of diethylaminoethanol on this arrhythmia was investigated. Eight cases of ventricular tachycardia were treated with the drug in various doses. In 6 cases there was prompt reversion to the rhythm which had been present before the tachycardia developed. The two in whom there was no response to the drug failed also to respond to other agents such as quinidine (both oral and intravenous), magnesium sulfate and digitalis (table III).

Five cases with auricular fibrillation showed no response to diethylaminoethanol in that normal rhythm was not resto[illegible] [illegible]nd the ventr[illegible]lar rate was not changed. The underlying heart disease i[illegible] [illegible] heart disease

TABLE III[3]

Treatment of ventricular tachycardia (VT) with intravenous diethylaminoethanol (DEAE)

	CASE 1—68 YRS MALE	CASE 2—41 YRS FEMALE	CASE 3—61 YRS MALE	CASE 4—74 YRS MALE
History	Heart failure for 3 yrs, etiology unknown Treated with digitalis salt poor diet, mercurial diuretics	Hypertension, arteriosclerotic heart disease	Arteriosclerotic and hypertensive cardiovascular disease with auricular fibrillation Treated with digitalis mercurial diuretics	Generalized arteriosclerosis
Acute episode	Auricular fibrillation and left hemiplegia Next day VT	Anterior wall infarction with cardiac failure Treated with digitoxin 1 2 mgm in 5 hrs 2 hrs later VT	Rapidly increasing heart failure Treated with 1 8 gms of digitalis Two days later VT	Coronary occlusion with left bundle branch block and ventricular premature contractions Next day VT
Treatment of VT prior to DEAE	None	Quinidine orally without effect	None	None
Treatment of VT with DEAE	2 grams DEAE Reversion to auricular fibrillation in 2 min VT returned in 9 min Additional 3 grams DEAE Reversion to auricular fibrillation VT did not recur	2 grams DEAE Normal sinus rhythm in 2 min VT returned in 25 min Additional 2 5 grams DEAE Normal sinus rhythm to date (2 months) (See fig 4)	5 grams DEAE Reversion to auricular fibrillation No recurrence of VT to date (2 months) (See fig 5)	8 grams DEAE Normal rhythm VT did not recur
Remarks	Died 12 days later from cerebral accident Autopsy large heart, moderate coronary arteriosclerosis	None	None	Died 4 hrs later Autopsy Fresh myocardial infarction of anterior wall
	CASE 5—55 YRS MALE	CASE 6—54 YRS MALE	CASE 7—60 YRS MALE	CASE 8—56 YRS MALE
History	Hypertensive cardiovascular disease with heart failure for 3 yrs Treated with digitalis mercurial diuretics	Hypertensive arteriosclerotic cardiovascular disease, left hemiplegia, diabetes mellitus Treated with protamine insulin and diet	Mild diabetes treated with diet Hypertensive heart disease, myocardial infarction with transient VT, several subsequent episodes of VT	Myocardial infarction 16 yrs ago
Acute episode	Abdominal cramps and bloody diarrhea then VT	Myocardial infarction Next day, shock and VT	Pulmonary edema with VT for 4 weeks	VT for 12 weeks with heart failure
Treatment of VT prior to DEAE	None	None	Quinidine quinine digitalis atabrine, magnesium sulfate without effect	Digitalis quinidine and atabrine orally and intravenously, quinine potassium chloride without effect
Treatment of VT with DEAE	5 grams DEAE Reversion to basic rhythm of auricular fibrillation No recurrence of VT to date (2 months)	6 grams DEAE Normal sinus rhythm VT returned shortly before death 12 hrs later	9 grams DEAE in divided doses without effect VT persisted uninterruptedly	8 grams DEAE in divided doses without effect VT persisted uninterruptedly.
Remarks	None	Autopsy not performed	Died 2 months later Autopsy not performed	Died shortly Autopsy not performed

[3] The first 6 cases were patients at Goldwater Memorial Hospital, New York, N. Y We are indebted to Dr Ernst Boas, Mt Sinai Hospital, New York, N. Y, for permission to report case 7 and to Dr S R Slater of the Jewish Hospital, Brooklyn, N Y, for case 8.

and in 2 arteriosclerosis. Four patients with supraventricular tachycardia also did not respond to diethylaminoethanol.

It was thought that the effect of diethylaminoethanol might be inherent in its hydroxyl group and that ethyl alcohol might have a similar action. However, the intravenous administration of 5 grams of ethyl alcohol in 5 per cent aqueous solution failed to abolish ectopic ventricular beats in 2 patients in whom the subsequent administration of diethylaminoethanol did result in suppression of the premature contractions.

As discussed above, the therapeutic effect of intravenous diethylaminoethanol on ventricular premature contractions was transitory, lasting in most cases less than 20 minutes. It is worthy of note that 20 minutes after intravenous administration (see Section I) the plasma levels of diethylaminoethanol were still considerably higher than the highest levels obtained after oral dosage (fig. 1). This suggests that the therapeutic effect of the drug on cardiac arrhythmias is dependent upon a very high plasma concentration which can only be achieved by intravenous administration. Whether this is also true for the other effects of the drug is not yet known.

Shortly after the injection of diethylaminoethanol, most subjects noted a peculiar taste variously described as bitter, metallic, or peppermint-like, followed by a sensation of warmth, dizziness and fluttering in front of the eyes. Nausea and vomiting were observed in about 15 per cent of the cases but did not occur until the change in rhythm had been effected. These side actions were usually transient and disappeared 10 to 15 minutes following the injection. In some instances a transitory fall in both the diastolic and systolic blood pressures was noted which disappeared within 20 minutes.

A few normal and a considerable number of hypertensive patients exhibited a marked fall in arterial pressure after receiving the drug. The fall in pressure was accentuated by the erect position. What limitation in the use of the drug this action may impose is being investigated.

SUMMARY

Diethylaminoethanol, a product of the hydrolysis, *in vivo*, of procaine, was studied in various cardiac arrhythmias. It protected dogs under cyclopropane anesthesia from the development of ventricular premature contractions and ventricular tachycardia. It suppressed ventricular premature contractions for a short period, in human subjects. Six cases of ventricular tachycardia were successfully reverted by the drug. No effect was noted on auricular fibrillation or supraventricular tachycardia.

Diethylaminoethanol is considerably less active than procaine. The effective dose however is much safer than the correspondingly effective dose of procaine.

The physiological disposition of diethylaminoethanol was studied in man. About 25 per cent of the drug was excreted in the urine; the remainder was metabolized by an unknown route. A single dose is almost completely metabolized or excreted in 8 hours. Considerable amounts of the compound are localized in the organs of the body (dog).

REFERENCES

1. Burstein, C. L., Marangoni, B. A., DeGraff, A. C., and Rovenstine, E. A. Anesthesiology, 1: 167, 1940.
2. Meek, W. J. Harvey Lect., 36: 188, 1940–41.
3. Burstein, C. L. Anesthesiology, 7: 113, 1946.
4. Brodie, B. B., Lief, P. A., and Poet, R. This Journal, *94*: 359, 1948.
5. Brodie, B. B., Udenfriend, S., and Baer, J. F. J. Biol. Chem., 168: 299, 1947.

THE ANTAGONISM OF CURARE BY CONGO RED AND RELATED COMPOUNDS

C. J. KENSLER[1]

Department of Pharmacology, Cornell University Medical College

Received for publication September 16, 1948

The revival of interest in curare and its current importance in medicine and surgery has made it desirable to investigate the anticurare activity of certain azo dyestuffs. The first observation that the injection of azo dyes into animals made it more difficult to curarize them was recorded by Petroff (1). Wetzloff (cited by Petroff) had observed the same action in the frog. Petroff (1, 2) found that congo red was the most effective of the dyes examined for activity in the frog and noted that it was active both prophylactically and therapeutically. The antagonism of crude curare preparations by congo red has also been observed by Hori (3), Hanzlik and Butt (4), Thienes (5), Ito (6) and Ishigami (7). Petroff (1, 2) suggested that the anticurare activity of congo red and related compounds was due to a reaction between curare and the colloidal azo dye micelle. This view was supported by a later report (8) that congo red formed a precipitate with crude curare.

More recently Richardson and Dillon (9) reported that congo red exerts a direct effect on cardiac and smooth muscle. Hanzlik (10) had observed that congo red increased the activity of smooth muscle, particularly intestine, *in vitro.* These observations suggest that congo red and related dyes may couple with tissue constituents and antagonize curare by a direct action on a functional component involved in neuromuscular transmission. The combination of Evans blue (T 1824) with serum proteins (11, 12) and the well established anticoagulant activity (13, 14, 15) of dyes of this type provide further basis for the speculation that the anticurare activity of congo red may be due to its combination with tissue components rather than to reactions occurring between the dye and the alkaloid.

In the present experiments the anticurare activity of congo red has been examined using pure d-tubocurarine chloride (DTC), which is a quaternary ammonium bisbenzylisoquinoline alkaloid (16, 17), and two pure erythrina alkaloids, beta-erythroidine·HCl (BE) and dihydro-beta-erythroidine·HCl (H_2BE), both tertiary ammonium alkaloids. Two preliminary reports on this work (18, 19) have been published.

METHODS. The compounds assayed for curare or anticurare activity were injected into the ventral lymph sac of the frog. The experiments were carried out at room temperature and three or more frogs were used in each group. Recovery from paralysis was arbitrarily defined as the recovery of the capacity of the frog to right itself five times consecutively when placed on its back. The duration of paralysis reported represents the period of time during which 50 per cent or more of the frogs in each group were paralyzed.

The experiments on the rectus abdominis muscle of the frog were carried out with the

[1] U. S. Public Health Research Fellow 1947.

isolated muscle using the technique of Chang and Gaddum (2) All experiments were done at room temperature The extent of contraction following test doses of acetylcholine was read off a millimeter rule at the end of two minutes

The cholinesterase activity measurements were carried out manometrically in a standard Warburg assembly The solution volume was 2 0 cc The medium was Ringer bicarbonate, the acetylcholine concentration was $M/50$, the temperature was 37 5°C , and the gas phase was 95 per cent O_2 and 5 per cent CO_2 The manometers were read every 10 minutes for a 30 minute period The results, per cent inhibition and normal activity, are based on the number of mm ^{3} of CO_2 produced in 30 minutes In the experiments in which human serum was used as the source of cholinesterase activity, the serum was diluted 1 to 5 with Ringer bicarbonate solution and 0 5 cc of the diluted serum added to the vessels In the case of the frog brain cholinesterase determination, 20 mgm of pooled whole frog brains homogenized by the technique of Potter and Elvehjem (21) were added to each vessel

The dialysis experiments were carried out using cellophane tubing obtained from the Visking Corp , Chicago, Ill The diameter of the inflated tubing was approximately 1 cm Most of the experiments were carried out in $M/15$ phosphate buffer, pH 7 4 The alkaloids or the azo dyes, or both, were placed inside the membrane and made up to a volume of 6 0 cc The cellophane tube was suspended in 44 cc of $M/15$ phosphate buffer in a beaker or graduated cylinder Aliquots were removed for turbidimetric (Valser's reagent) or biologic (paralysis of frogs) assay to determine the extent of diffusion of the alkaloids through the membrane These experiments were done at room temperature

Valser's reagent, as described by Travell (22) has been found satisfactory for the determination of all three alkaloids providing the turbidity of the solution is read within 15 seconds Six cc of the solution to be tested and 0 3 cc of Valser's reagent were mixed and the resulting turbidity was measured in a Klett Summerson colorimeter The standards as well as the dialysis experiment aliquots were run in $M/15$ phosphate buffer, pH 7 4 BE formed the least stable suspension with Valser's reagent, flocculation of the suspension decreased the reading by as much as 50 per cent in 2 to 3 minutes The limit of sensitivity under these conditions was 15 micrograms/cc for DTC, 20 micrograms/cc for H_2BE, and 100 micrograms/cc for BE The Klett Summerson colorimeter readings (a logarithmic scale) were a linear function of the concentration of the alkaloids in the range examined above the lower limiting concentration However, as BE and H_2BE give progressively lower values as a function of the time they are kept in phosphate buffer, pH 7 4, the turbidimetric results in the dialysis experiments are given in Klett Summerson scale units rather than micrograms/cc

RESULTS *In vivo Duration of paralysis* As is shown in table I, the duration of paralysis following the injection of DTC into the ventral lymph sac of the frog is roughly proportional to the dose in amounts above 2 mgm /kgm Paralysis of 50 per cent of the frogs has not been observed with amounts of 2 mgm /kgm or less As is indicated in the last column of this table, the variation from experiment to experiment was quite marked However, agreement between the duration of paralysis at a constant dose of DTC for several groups of frogs from the same batch observed at the same time was good

Unna, Kniazuk and Greslin (23) found that the duration of paralysis following the injection of the erythrina alkaloids into the ventral lymph sac of the frog was roughly proportional to the dose The data obtained in the present experiments are in good agreement with the reported values Six mgm /kgm of BE and 0 5 mgm /kgm of H_2BE were found to paralyze the frogs for two hours The data indicate that BE is slightly less active than DTC and that H_2BE is several times as potent when judged on the basis of the duration of paralysis in the frog

However, in other experiments carried out in this laboratory using the rat phrenic nerve-diaphragm preparation of Bülbring (24), H_2BE was found to be only $\frac{1}{10}$ as active as DTC and BE less than $\frac{1}{30}$ as active.

TABLE I

Duration of paralysis in frogs produced by d-tubocurarine

D-TUBOCURARINE DOSE	NUMBER OF EXPERIMENTS	NUMBER OF FROGS	DURATION OF PARALYSIS	VARIATION
mgm./kgm.			*hours*	*hours*
2	2	10	0	0
3	2	10	0.5	0.4–0.6
5	8	24	3.0	2.0–4.0
10	17	51	6.0	4.0–7.0

TABLE II

The anticurare activity of azo dyes in frogs subsequently injected with d-tubocurarine chloride

EXP.	DOSE D-TUBOCURARINE	DURATION PARALYSIS CONTROLS*	TREATMENT COMPOUND 200 MGM./KGM.	INTERVAL BETWEEN INJECTION OF DYE AND ALKALOID	NUMBER OF FROGS PARALYZED	DURATION OF PARALYSIS	INHIBITION OF PARALYSIS
	mgm./kgm.	*hours*		*hours*		*hours*	*per cent*
1	5	3.0	Congo red	1	0/4	0	100
2	5	3.2	Congo red	1	0/4	0	100
3	5	3.5	Congo red	24	0/4	0	100
4	5	4.0	Congo red	24	0/4	0	100
5	5	2.5	Congo red	24	0/4	0	100
2	5	3.2	Chlorazol fast pink	1	0/4	0	100
5	5	2.5	Chlorazol fast pink	24	0/4	0	100
6	10	5.0	Evans blue	1	0/4	0	100
7	10	6.5	Evans blue	1	0/4	0	100
6	10	5.0	Methyl orange	1	4/4	5.5	0
7	10	6.5	Methyl orange	1	4/4	6.0	7
1	5	3.0	Bis J acid urea	1	0/4	0	100
2	5	3.2	Bis J acid urea	1	0/4	0	100
3	5	3.5	Bis J acid urea	24	4/4	1.2	67
4	5	4.0	Bis J acid urea	24	4/4	3.0	25
5	5	2.5	Bis J acid urea	24	4/4	1.0	60
8	5	3.0	DFP	1	4/4	1.5	50
9	5	4.0	DFP	24	4/4	2.0	50
10	5	3.0	DFP	24	4/4	1.5	50

* All frogs paralyzed.

Prophylactic anticurare activity of azo dyes in the frog. Experiments carried out with both Intocostrin and pure DTC provided evidence that congo red and the related dyes, Evans blue and chlorazol fast pink, prevent paralysis when doses up to 10 mgm./kgm. of DTC are used. Thus, the observations of the earlier

workers on the anticurare activity of congo red have been confirmed and extended to the pure alkaloid DTC. The data obtained on DTC are summarized in table II.

The results of a typical experiment are plotted in figure 1. Paralysis (see definition under methods) is plotted as a function of time. Congo red gave complete protection, whereas when diisopropylfluorophosphate (DFP) was used the duration of paralysis was approximately 50 per cent of that in the controls, i.e. per cent inhibition by DFP equals 50 per cent.

From the data presented in table II it is clear that congo red and chlorazol fast pink afford good protection even 24 hours after injection. Congo red- and chlorazol fast pink-treated frogs were also tested 6 days after injection and a protective effect was observed although it was not complete. One compound which

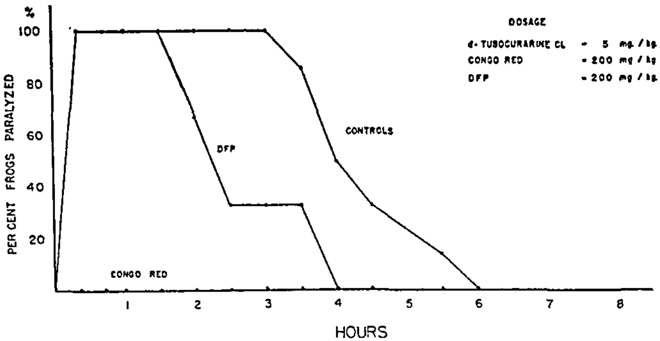


FIG. 1. PROPHYLACTIC ANTICURARE ACTIVITY OF CONGO RED AND DIISOPROPYL FLUOROPHOSPHATE (DFP)

does not contain an azo linkage, bis J acid urea, has been found to be effective. However, as is indicated in table II, its anticurare activity wears off much more rapidly than that of the azo dyes. Whether this difference is due to increased excretion or destruction or to the possibility that it is less active on a weight basis than the azo dyes has not been established. Methyl orange, a mono sulfonic acid azo dye, was essentially inactive, although paralysis was delayed by a half-hour. DFP, which is known to be a potent irreversible inactivator of cholinesterase, showed some anticurare activity, although not as much as that exhibited by the azo dyes.

In marked contrast to the protection afforded by the azo dyes against DTC, no protective action was observed when the pure erythrina alkaloids, BE or H_2BE, were used as the paralyzing agent. The data obtained using congo red and bis J acid urea are shown in table III. As these experiments with the erythrina alkaloids were done in July and August with "summer" frogs and the experiments with DTC had been done with "winter" frogs, the frogs used in experiments 4

and 6, table III, were injected 24 hours later with a paralyzing dose of DTC. As was expected, in each case, the control frogs were paralyzed and the congo red-injected frogs were not. These experiments thus rule out the possibility that the failure of congo red to prevent paralysis by the erythrina alkaloids was due to seasonal variation either in the frogs or the temperature prevailing when the

TABLE III

Protection against paralytic doses of the erythrina alkaloids

EXP. NO.	ALKALOID	DOSE	DYE	DOSE	NUMBER OF FROGS PARALYZED	DURATION OF PARALYSIS	DECREASE IN DURATION OF PARALYSIS
		mgm./kgm.		*mgm./kgm.*		*hours*	*per cent*
I	BE*	3	—	—	6/6	1.0	—
	BE*	3	Congo red	200 (1)‡	4/4	1.0	0
II	BE	6	—	—	4/4	2.0	—
	BE	6	Congo red	200 (1)	4.4	2.5	0
III	H_2BE†	2	—	—	4/4	7.0	—
	H_2BE†	2	Congo red	200 (1)	4/4	8.0	0
IV	H_2BE	0.5	—	—	4/4	2.0	—
	H_2BE	0.5	Congo red	200 (1)	4/4	2.5	0
V	H_2BE	1	—	—	4/4	4.5	—
	H_2BE	1	Bis J acid urea	200 (1)	4/4	4.0	11
VI	H_2BE	0.5	—	—	6/6	1.2	—
	H_2BE	0.5	Congo red	200 (1)	6/6	1.0	16
	H_2BE	0.5	Bis J acid urea	200 (1)	6/6	1.0	16
VII	BE	4	—	—	6/6	1.4	—
	BE	4	DFP	200 (1.2)	3/6	0.8	45
VIII	BE	4	—	—	6/6	1.3	—
	BE	4	DFP	200 (1.5)	3/6	0.4	70
IX	BE	4	—	—	6/6	2.0	—
	BE	4	DFP	100 (3)	6/6	1.5	25

* Beta-erythroidine·HCl.
† Dihydro-beta-erythroidine·HCl.
‡ Time interval between injection of dye and the erythrina alkaloids in hours.

experiments were run. It should also be pointed out that the dose levels of the erythrina alkaloids and DTC were approximately equal in so far as duration of paralysis is concerned. Hence, there is a striking difference in the anticurare activity of congo red which is dependent on the paralytic substance used. Unna, Kniazuk and Greslin (23) have shown in the mouse that physostigmine and neostigmine are as effective antagonists for the erythrina alkaloids as they are

for DTC (Unna and Kimura, 25) in contrast to this difference in action of the azo dyes in the frog. The data in tables II and III show that in the frog DFP exerts some protective action against both DTC and BE.

In view of the failure of congo red to prevent paralysis by the erythrina alkaloids in the frog, it was thought desirable to determine whether DTC and one of the erythrina alkaloids H_2BE were additive in their curarizing activity. If this were found to be the case, it would constitute suggestive evidence that DTC and H_2BE were acting at the same physiological site. In the four experiments performed, DTC and H_2BE were found to have an additive effect. In each experiment the administration of DTC and H_2BE paralyzed the frogs for longer periods than either one alone. In one experiment amounts of each sufficient to paralyze only 1 out of 4 frogs, when combined paralyzed 4/4 for an average period of 90 minutes.

Petroff (1) reported that congo red exerted an anticurare action in the dog. In the present experiments the prophylactic anticurare activity of bis J acid urea and chlorazol fast pink has been examined in the cat. The LD_{50} for intravenously administered DTC based on the use of 25 cats is approximately 2.5 units/kgm. Data obtained from 10 cats indicate that bis J acid urea and chlorazol fast pink increase the LD_{50} by 60 to 100 per cent. This increase is similar in magnitude to the 100 per cent increase in the LD_{50} reported by Unna and Kimura (25) in mice when physostigmine or neostigmine were used prophylactically. The prophylactic anticurare action of bis J acid urea and chlorazol fast pink was also shown by the increased survival time of those treated cats that died. However, paralysis was not prevented completely in any of the treated animals.

Therapeutic anticurare activity of azo dyes and related compounds. The ability of congo red and related compounds to increase the rate of recovery of frogs paralyzed with DTC is shown in tables IV and V. The dyes were injected 30 minutes after the alkaloid, when all the frogs were paralyzed. Congo red, chlorazol fast pink and dichlorazol blue (Chicago blue) were found to be effective. As all the azo dyes which had been found effective by Petroff (1, 2) and those used in these experiments contain naphthalene sulfonic acid groups, a number of simpler compounds of this type were tested. The only one which showed significant activity was bis J acid urea. This compound does not contain any azo linkages and is not highly colored. J acid, as well as all of the other naphthalene sulfonic acid compounds listed in table VI failed to decrease the recovery time of frogs paralyzed with DTC. Thus the anticurare activity of the naphthalene sulfonic compounds appears to be limited to those of relatively high molecular weight. The plot of a typical therapeutic experiment is shown in figure 2.

In vitro experiments with the frog rectus abdominis muscle of the frog. Acetylcholine is capable of initiating a contraction of the isolated rectus abdominis muscle of the frog. Under standard conditions the degree of shortening is roughly proportional to the concentration of acetylcholine. Chang and Gaddum (20) have made this response the basis of a biological assay for acetylcholine. The response of this muscle to acetylcholine can be blocked by curare (26, 27, 28). These observations suggested the use of this *in vitro* muscle preparation to study the

antagonism between congo red and DTC and H_2BE. In the present experiments both DTC (8 to 32 micrograms/cc.) and H_2BE (5 to 15 micrograms/cc.) have

TABLE IV

The effect of various compounds on the recovery of frogs paralyzed with ca. 10 mgm./kgm. of d-tubocurarine chloride

COMPOUND*	RECOVERY TIME† CONTROLS	RECOVERY TIME TREATED	RECOVERY OF TREATED FROGS AS PER CENT OF CONTROL TIME
	hours	*hours*	*per cent*
Congo red	5.2	0.9	17
Congo red	7.0	2.5	36
Congo red	7.0	3.0	43
Congo red	7.0	3.0	43
Congo red	6.0	1.0	17
Congo red	4.8	1.8	37
Congo red	6.0	2.0	33
			Average 32
Chlorazol fast pink	5.5	0.8	15
Chlorazol fast pink	4.0	1.0	25
Chlorazol fast pink	7.0	3.5	50
Dichlorazol	7.0	2.5	36
Dichlorazol	7.0	4.0	57
2,2′ dihydroxy-5,5′-disulfonic acid-azo-benzene	7.0	7.2	103
Diisopropyl fluorophosphate (200 mgm./kgm.)	6.0	3.0	50
Diisopropyl fluorophosphate (200 mgm./kgm.)	4.8	2.0	42
Diisopropyl fluorophosphate (200 mgm./kgm.)	6.0	5.0	83
Diisopropyl fluorophosphate (200 mgm./kgm.)	5.0	3.0	60
β naphthol-4-sulfonic acid	5.6	5.6	100
Flavianic acid	5.6	4.2	75
Flavianic acid	4.3	4.0	93
Sulphanilic acid	7.0	7.0	100
Sulphanilic acid	6.5	6.3	97
Chromotropic acid	6.2	4.3	70
Chromotropic acid	6.5	6.5	100
Chromotropic acid	5.6	5.6	100

* Dose of all compounds ca. 200 mgm./kgm.
† Three or more frogs were used in each determination.

been found to block the response of the rectus muscle to acetylcholine (0.75 to 2.0 micrograms/cc.).

The exposure of this muscle preparation to congo red (0.5 mgm./cc.) for 60 to 90 minutes was observed to potentiate the response to a standard dose of acetyl-

choline and also to decrease the threshold concentration of acetylcholine required to produce a contraction. This potentiation is shown in the data included in

TABLE V

The effect of various compounds on the recovery of frogs paralyzed with ca. 5 mgm./kgm. of d-tubocurarine chloride

COMPOUND*	RECOVERY TIME† CONTROLS	RECOVERY TIME TREATED	RECOVERY OF TREATED FROGS AS PER CENT OF CONTROL TIME
	hours	*hours*	*per cent*
Congo red	3.0	1.0	33
Congo red	3 2	1 3	40
Congo red	2.0	0.5	25
Congo red	3 7	1.3	35
Congo red	>4.0	0.8	<20
Congo red	3.2	1.7	53
Congo red	2.5	0.8	33
Congo red	2.6	0 7	27
			Average 33
1,3 bis J acid urea	3.0	0.7	23
1,3 bis J acid urea	3 2	1.0	31
1,3 bis J acid urea	2 0	0.7	35
1,3 bis J acid urea	>4.0	0.6	<15
			Average 26

* Compounds injected in amounts ca 200 mgm /kgm.

† Three or more frogs per experiment

TABLE VI

Additional compounds which did not decrease the duration of paralysis by d-tubocurarine chloride

1 1-naphthylamine 2-sulfonic acid
2. 1-naphthylamine-4 sulfonic acid
3. 1-naphthylamine-5-sulfonic acid
4 1-naphthylamine-6-sulfonic acid
5 1-naphthylamine-7-sulfonic acid
6 1-naphthylamine-8-sulfonic acid
7. 2-naphthylamine-1-sulfonic acid
8. 2-naphthylamine-6-sulfonic acid
9. 2-naphthylamine-5,7-disulfonic acid
10. 2-naphthylamine-1,6 disulfonic acid
11. 1-amino-2-naphthol-4-sulfonic acid
12. 2-amino-8-naphthol-6-sulfonic acid
13. 1-amino 8-naphthol-6-sulfonic acid
14. 2-amino-5-naphthol-7-sulfónic acid (J acid)

tables VII and VIII. Congo red was not observed to exert any direct action on the muscle although it was stained red and repeated washings failed to remove the dye. As is shown in table VII, when the muscle was maintained in the congo

red solution DTC failed to exert its expected blocking action. It can also be seen that even if as much congo red as will wash out has been removed from the muscle, some protection against the blocking action of DTC remains.

In contrast to the protection afforded by congo red against DTC, when the erythrina alkaloid H_2BE was used complete inhibition of the acetylcholine response was obtained even though congo red was present throughout the exposure to the alkaloid. The data are shown in table VIII. Thus, the data obtained using the rectus muscle preparation are in harmony with the results in the frog *in vivo*, namely, that protection is afforded by congo red against DTC but not against H_2BE.

The observation that congo red is capable of potentiating the response of the rectus to acetylcholine suggested the possibility that congo red may possess

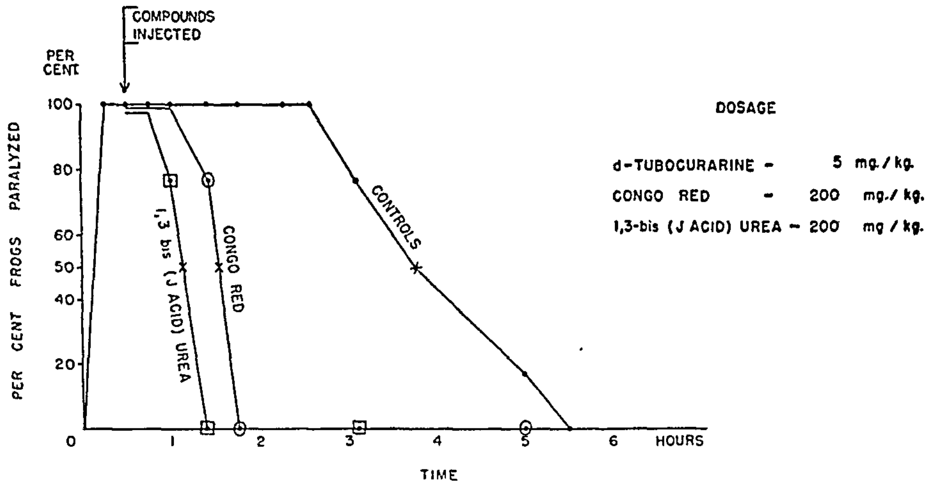


FIG. 2. THE EFFECT OF CONGO RED AND 1,3-BIS (J ACID) UREA ON THE RECOVERY OF FROGS PARALYZED BY D-TUBOCURARINE

anticholinesterase activity. The anticurare activity of inhibitors of cholinesterase made it of importance to investigate this possibility.

The anticholinesterase activity of azo dyes. Massard and DuFait (29) reported that sulfonic acid azo dyes do not inhibit the cholinesterase activity of horse serum. The highest concentration of congo red employed in their experiments was 2×10^{-4} M. It is known that cholinesterase from different sources is inhibited to a different extent by the same concentration of inhibitor (30). In view of this fact, as well as the observations on the rectus muscle, congo red and related compounds have been examined as cholinesterase inhibitors using human serum and frog brain as the source of the enzyme.

The data obtained on the anticholinesterase activity of congo red and related compounds are summarized in table IX. Congo red has been found to be a moderately potent inhibitor of frog brain cholinesterase activity. However, DFP is five hundred times more active. The data on Chicago blue, chlorazol fast pink

TABLE VII

Prevention by congo red of the blocking action of d tubocurarine on the response of the frog rectus to acetylcholine

EXP	TEST DOSE OF ACETYLCHOLINE	CONC OF CONGO RED	DURATION OF EXPOSURE TO CONGO RED	CONC OF D TUBOCURARINE	DURATION OF EXPOSURE TO D-TUBOCURARINE	RANGE OF RESPONSE OF THE MUSCLE	INHIBITION BY D TUBOCURARINE
	micro grams/cc	*mgm /cc*	*minutes*	*micro grams/cc*	*minutes*	*mm*	*per cent*
1	0 75	—	—	—	—	4 0– 8 0	
	0 75	0 50	70	—	—	14 0–15 0	
	0 75	0 50	100	33	30	14 0–19 0	0
2	0 75	—	—	—	—	2 0– 3 0	
	0 75	0 50	60	—	—	6 0– 6 0	
	0 75	—	—	33	30	4 0– 5 0	25
3	1 5	—	—	—	—	1 0– 3 0	
	1 5	0 50	60	—	—	6 0– 7 5	
	1 5	—	—	33	30	2 0– 3 0	63
4	0 75	—	—	—	—	12 0–19 0	
	0 75	—	—	8	30	0	(100)*
	30 minutes later after rinsing						
	0 25	—	—	—	—	10 5–11 5	
	0 25	0 50	62	—	—	17 5–21 5	
	0 25	—	—	8	31	19 0–21 5	0

* Before exposure to congo red

TABLE VIII

Failure of congo red to prevent the blocking action of dihydro beta erythroidine on the response of the frog rectus to acetylcholine

EXP	TEST DOSE OF ACETYLCHOLINE	CONC OF CONGO RED	DURATION OF EXPOSURE TO CONGO RED	CONC OF DIHYDRO BETA ERYTHROIDINE	DURATION OF EXPOSURE TO DIHYDRO BETA ERYTHROIDINE	RANGE OF RESPONSE OF THE MUSCLE	INHIBITION BY DIHYDRO BETA ERYTHROIDINE
	micro grams/cc	*mgm /cc*	*minutes*	*micro grams/cc*	*minutes*	*mm*	*per cent*
1	1 5	—	—	—	—	2 – 3	
	2 5	—	—	—	—	6 5– 8	
	1 5	0 5	100	—	—	14 –16	
	2 5	0 5	100	—	—	19 –21	
	1 5	0 5	115	12	15	0	100
	2 5	0 5	115	12	15	0	100
2	1 5	—	—	—	—	10 –11	
	1 5	0 5	96	—	—	24 –26	
	1 5	0 5	111	12	15	0	100
3	3 0	—	—	—	—	3 – 4	
	3 0	0 5	90	—	—	8 –10	
	3 0	0 5	105	5	15	0	100

and bis J acid urea show a wide range in the anticholinesterase activity of the effective anticurare sulfonic acid compounds. Chlorazol fast pink has very little anticholinesterase activity except at extremely high concentrations and bis J acid urea is only one four hundredths ($\frac{1}{400}$) as active as congo red.

Congo red (the azo dye with the highest anticholinesterase activity *in vitro*) and DFP, in doses of 200 mgm./kgm. have been examined for an effect on frog brain cholinesterase activity *in vivo*. Whereas DFP was found to inhibit 70 to 75 per cent of brain cholinesterase activity, congo red did not measurably inhibit activity.

Thus, the investigation of the cholinesterase inhibiting properties of the effective anticurare azo dyes has shown that several of the dyes are moderately active inhibitors *in vitro*. However, the lack of any obvious correlation between cholinesterase inhibiting activity and anticurare activity, the lack of effect on brain cholinesterase activity *in vivo*, and the failure to exert an anticurare activity when the erythrina alkaloids were employed, indicated that the anticurare activity of the azo dyes is not due chiefly to their anticholinesterase activity.

TABLE IX

The concentration of congo red and related compounds required to inhibit 50% of the cholinesterase activity of human serum and frog brain in vitro

COMPOUND	FROG BRAIN	HUMAN SERUM
Diisopropyl fluorophosphate	1.7×10^{-7} *M*	8×10^{-8} *M*
Congo red	7.5×10^{-5} *M*	9×10^{-4} *M*
Evans blue	9.0×10^{-5} *M*	—
Chicago blue	2.0×10^{-4} *M*	—
1,3-bis(J acid) urea	1.8×10^{-3} *M*	$>1 \times 10^{-2}$ *M*
Chlorazol fast pink	$>1 \times 10^{-2}$ *M*	—

Evidence for a direct combination between congo red and DTC. It has been observed, in agreement with Petroff's (8) observation with crude curare, that congo red and DTC will, under certain conditions, form a heavy red precipitate. An investigation of this phenomenon has disclosed that this precipitate is formed at a neutral pH in water and also occurs in *M*/15 phosphate buffer, pH 7.4. It has been found that the mole ratio of DTC to congo red is the determining factor in the formation of the precipitate. If the mole ratio of DTC to congo red is less than 1:4, no precipitate will form. As the mole ratio is increased to 1:2, the bulk of the congo red is precipitated out of solution. Data illustrative of this point are shown in table X. The amount of congo red in solution after the precipitate had been centrifuged off was measured colorimetrically and the results are expressed in Klett-Summerson colorimeter scale units. The initial concentration of congo red was 0.01 per cent.

At a mole ratio of 1:1 congo red is completely precipitated out of solution and examination of the supernatant solution by both frog assay *in vivo* and turbidimetric assay with Valser's reagent showed no detectable DTC to be present. The limiting sensitivity of these methods makes it possible to state that at least

75 per cent of the DTC on the basis of the frog assay and 90 per cent on the basis of the turbidity test had been removed from the colorless supernatant solution by the formation of the precipitate The observation of Hanzlik (31) that congo red prevents the formation of a precipitate when Mayer's reagent (a mercuric iodide reagent as is Valser's) is added to several alkaloids has been confirmed in the case of DTC and Valser's reagent However, this effect of congo red does not influence the result of the turbidimetric assay as all of the congo red had been precipitated from the solution by the addition of DTC Thus, both the congo red and DTC are contained in the precipitate, which indicates that some type of combination has occurred and that the precipitate is not simply a "salting out" of the colloidal dye Further evidence of the formation of a complex between DTC and congo red is provided by the observation that this precipitate can be dissolved in excess congo red Excess DTC will not dissolve the precipitate

TABLE X

Precipitation of congo red by d tubocurarine chloride in phosphate buffer pH 7 4

MOLE RATIO D-TUBOCURARINE / CONGO RED	CONGO RED REMAINING IN SOLUTION KLETT SUMMERSON SCALE UNITS	
	Experiment 1	Experiment 2
0	1400	1350
0 13	1420	1330
0 25	—	1280
0 40	1220	—
0 50	500	600
0 60	—	40
0 75	10	20

An examination of Evans blue, Chicago blue, chlorazol fast pink and bis J acid urea has shown that the mole ratio of DTC to the dye required to produce a precipitate varies among these compounds A mole ratio of 4:1 is required to precipitate Evans blue Chicago blue and chlorazol fast pink are in the same range as Evans blue and bis J acid urea is in the same range as congo red Evans blue, chlorazol fast pink and Chicago blue contain more sulfonic acid groups per molecule than do congo red and bis J acid urea It appears likely that the formation of a precipitate is due to the formation of salt linkages between the alkaloid and the sulfonic acid groups of the dye molecules

The formation of a precipitate by the combination of DTC and a sulfonic acid compound is not limited to the colloidal sulfonic acid azo dyes For example, methyl orange, a mono azo compound having one sulfonic acid group, also forms a precipitate with DTC This compound, as mentioned previously, did not significantly decrease the duration of paralysis produced by DTC although its induction was delayed

The use of the erythrina alkaloids in place of DTC has shown that BE and H_2BE up to mole ratios of 20:1 do not form a precipitate with congo red

The formation of an insoluble complex between DTC and congo red and its

reversal by the addition of excess congo red suggest that DTC forms a soluble complex with dyes of this type. Measurement of the absorption spectrum of congo red in the visible region in a Beckman spectrophotometer did not show any

TABLE XI

Dialysis experiments with d-tubocurarine chloride and azo dyes

EXP. NO.	INSIDE MEMBRANE*		PERIOD OF DIALYSIS	PASSAGE OF D-TUBOCURARINE THROUGH MEMBRANE	
	Amount of d-tubocurarine	Dye		Frog assay†	Turbidimetric assay
	mgm.	*mgm.*	*hours*	*hours*	*micrograms/cc.*
I	10	—	2.5	2.0	—
	10	—	18.	4.0	—
II	10	—	3.5	4.0	—
	10	Congo red 10	3.5	0	—
	10	Evans blue 10	3.5	0	—
III	10	—	22.	3.0	—
	(Aliquot from inside membrane)		22.	3.5	—
	10	Congo red 10	22.	0	—
	10	Evans blue 10	22.	0	—
IV	10	Methyl orange 10	20.	2.5	—
V	10	Methyl orange 10	22	3.5	—
VI	1	—	1.5	—	10
	1	—	19.	—	20
	1	Congo red 10	1.5	—	0
	1	Congo red 10	19.	—	0
VII	2	—	1.	—	Trace
	2	—	4.	—	20
	2	—	24.	—	36
	2	—	60.	—	36
	2	Bis J acid urea 10	1.	—	0
	2	Bis J acid urea 10	4.	—	0
	2	Bis J acid urea 10	24.	—	Trace
	2	Bis J acid urea 10	60.	—	Trace

* 5.0 to 6.0 cc. $M/15$ phosphate buffer pH 7.4 inside membrane. 44.0 to 45.0 cc. $M/15$ phosphate buffer pH 7.4 outside membrane.

† 0.5 cc. aliquot from outside the membrane (except as noted in Exp. III) was injected into 3 to 6 frogs.

shift in the absorption curve when small amounts of DTC were added. It was thought that if such a complex was formed its presence could be demonstrated by the use of a membrane impermeable to congo red and related dyes but permeable to DTC.

Dialysis experiments. The results of the dialysis experiments are summarized

in tables XI and XII. DTC and the erythrina alkaloids passed through the cellophane membrane when dissolved in phosphate buffer, pH 7 4, while congo red and related dyes did not When DTC and the azo dyes were both placed inside the membrane, DTC no longer diffused through Methyl orange, which forms a yellow precipitate with DTC, did not prevent the diffusion of DTC through the membrane although its diffusion was slower

TABLE XII

Dialysis experiments with the erythrina alkaloids and congo red

EXP NO	INSIDE MEMBRANE*		CONGO RED	PERIOD OF DIALYSIS	DURATION OF PARALYSIS	TURBIDIMETRIC ASSAY†
	Beta erythroidine	Dihydro-beta erythroidine				
	mgm	*mgm*	*mgm*	*hours*	*hours*	
I	—	5	—	2	—	425
	—	5	—	4	—	.550
	—	5	20	2	—	500
	—	5	20	4	—	500
II	5	—	—	2	—	2
	5	—	—	4	—	14
	5	—	20	2	—	4
	5	—	20	4	—	20
III	—	5	—	2	4 0	700
	—	5	20	2	3 5	660
IV	—	5	—	2	3 5	580
	—	5	20	2	3 5	600
V	10	—	—	3	0 5	—
	10	—	20	3	0 7	—

* 7 0 cc $M/15$ phosphate buffer pH 7 4 inside membrane 23 0 cc $M/15$ phosphate buffer pH 7 4 outside membrane

† Klett Summerson Scale Readings

In contrast to the prevention of DTC diffusion through the membrane by the azo dyes, the diffusion of the erythrina alkaloids was not noticeably altered The mole ratio of congo red to the erythrina alkaloids was approximately 2:1 in these experiments In the dialysis experiments with congo red and DTC a mole ratio of 1.1 as well as 10.1 prevented the passage of DTC through the membrane

Thus the dialysis experiments with DTC and the sulfonic acid compounds indicate that they form a non dialyzable complex and that the erythrina alkaloids do not form one Hence, the results of the dialysis experiments support the hypothesis that congo red and related compounds owe their anticurare (DTC) activity chiefly to a reaction occurring between the alkaloid and the dye rather than to the action of the dye on a functional tissue component involved in neuromuscular transmission

Discussion. The data presented in the preceding sections clearly demonstrate that congo red and related compounds possess anticurare activity in the frog when d-tubocurarine (DTC) is the curarizing alkaloid, and that no such activity can be detected when the erythrina alkaloids, beta-erythroidine (BE) and dihydro-beta-erythroidine (H_2BE) are used. DFP exerted some anticurare activity against both DTC and BE. Physostigmine and neostigmine are known to antagonize DTC in other species and Unna, Kniazuk and Greslin (25) have found this to be true for the erythrina alkaloids as well. Thus it appears that the sulfonic acid azo dyes are effective anti-DTC agents by some other mechanism than that (or those) involved in the anticurare activity of neostigmine, physostigmine and DFP.

The anticurare activity of DFP is presumably due to its anticholinesterase activity, and while both neostigmine and physostigmine are potent anticholinesterases, there is evidence that this action is not entirely due to anticholinesterase activity (32). The observation that several of the azo dyes are moderately potent cholinesterase inhibitors suggests that this action might be the basis of their anti-DTC activity. However, their failure to protect against the erythrina alkaloids and the practically indetectable anticholinesterase activity of chlorazol fast pink, both tend to rule out this phenomenon as the basic action on which the anti-DTC activity is dependent.

In so far as is known both DTC and the erythrina alkaloids block neuromuscular transmission by acting at the same site. The lack of precise knowledge of the details of processes involved in transmission through the neuromyal junction makes it apparent that this is an assumption which may or may not be justified, even though in a broad sense their pharmacologic action in this area is the same. The observation that the tertiary amine, BE, and the quaternary amine, DTC, are additive in their curarizing activity, at least in the frog, also supports this assumption. As there is no evidence that the erythrina alkaloids act in a different manner, the assumption that they are acting on the same functional tissue component appears justified. This reasoning leads one to suspect that the anti-DTC activity of the azo dyes is due to their prevention of the DTC from reaching, or its removal from, its site of action. Evidence that DTC reacts with congo red and related compounds to form a non-dialyzable complex has been presented. Under similar conditions no such complex formation was noted in the case of the erythrina alkaloids and congo red. The evidence thus indicates that the anti-DTC activity of congo red and related compounds is due chiefly to this reaction, although the anticholinesterase activity may contribute to this action in the case of some of the compounds.

The observation that more DTC is required to precipitate equivalent amounts of Evans blue and chlorazol fast pink than congo red and bis J acid urea may be due to the number of sulfonic acid groups on these molecules. Evans blue and chlorazol fast pink have four, whereas congo red and bis J acid urea each have two. The formation of a precipitate of DTC and methyl orange, a monosulfonic acid dye, in less than a 1:2 mole ratio suggests that the interaction of DTC and the sulfonic acid groups of the dyes is involved in the reaction. The data suggest that as the sulfonic acid groups of these molecules are successively bound, pre-

sumably in a salt linkage, with DTC, the solubility of the dye micelle is decreased and finally results in a precipitate.

The data presented in tables IV and V indicate that the congo red accelerated rate of recovery of frogs paralyzed with both the 5 and 10 mgm./kgm. dose of DTC remained the same when expressed as a percentile function of the duration of paralysis in the controls. The dose of congo red was the same in both cases and represented a mole ratio of approximately 40 to 1 and 20 to 1, respectively. As the congo red is present in large excess in both cases this suggests that the limiting reaction is the dissociation rate of the DTC-tissue component complex rather than the reaction between the alkaloid and the dye.

The hypothesis that effective anticurare agents might be of value in the treatment of myasthenia gravis (33) led to the use of neostigmine in the treatment of this disease. Similar reasoning has led to the use of congo red in the treatment of this disease (34). Only a few patients were treated and some improvement was reported. The results obtained in the experiments reported in the paper indicate that congo red, apart from its anticholinesterase activity, would be effective only if the production of an agent similar to DTC rather than to BE were the cause of the paralysis encountered in these patients. Thus, the further investigation of Ravin's preliminary report on congo red might be useful from the standpoint of gaining some understanding of the disease.

SUMMARY

Congo red and related compounds prevent paralysis in the frog when d-tubocurarine chloride is the curarizing agent, but not when beta-erythroidine·HCl or dihydro-beta-erythroidine·HCl is the curarizing agent. Congo red also shortens the recovery time of frogs paralyzed with d-tubocurarine chloride.

Congo red prevents the inhibition by d-tubocurarine of the response of the isolated frog rectus abdominis muscle to acetylcholine. The inhibition by the erythrina alkaloids is not prevented. Incubation with congo red potentiates the response of the frog rectus muscle to acetylcholine.

Congo red is a moderately potent inhibitor of frog brain cholinesterase activity *in vitro* but no inhibition was observed *in vivo*. Related compounds varied in their anticholinesterase activity and there was no apparent correlation between this property and their anti-d-tubocurarine activity.

Congo red and related compounds prevent the passage of d-tubocurarine through a cellophane membrane but do not affect the passage of the erythrina alkaloids. Additional evidence, i.e. precipitate formation, is presented which indicates that d-tubocurarine and congo red form a complex which is soluble or insoluble depending on the mole ratio of d-tubocurarine to congo red.

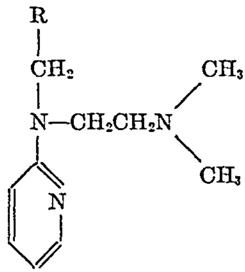
The evidence which supports the hypothesis that congo red and related compounds are effective against d-tubocurarine because of a reaction with d-tubocurarine rather than an action on a functional component of the myoneural junction is discussed.

Acknowledgment. The author is indebted to Dr. Newcomer of Squibb and Sons for the d-tubocurarine chloride, to Dr. Major of Merck and Co. for the

Action on the isolated guinea pig uterus. The antihistaminic drugs described here were found to stimulate the isolated uterus. The minimal stimulating doses were: WIN 2848—6.3, WIN 2875—5.7, WIN 2876—5.7, and tripelennamine—6.3. Histamine stimulated the isolated uterus at these dilutions. The antihistaminic agents used in this investigation do not antagonize this contracture and appear to be histamine-like on the isolated guinea pig uterus.

TABLE 1

Spasmolytic action on the isolated intestinal segment



DRUG	R	SPASMOLYTIC POTENCY*	
		Histamine contracture (guinea pig ileum)	Acetylcholine contracture (rabbit ileum)
WIN 2848	3-thienyl	8.3	6.0
WIN 2875	2-chloro-3-thienyl	7.2	5.3
WIN 2876	2-bromo-3-thienyl	7.9	5.3
Thenylpyramine†	2-thienyl	7.7	6.5
Tripelennamine‡	phenyl	7.4	6.0

* *Expressed as the log of the effective dilution as determined by Miller, Tainter and* Becker (6). Values represent concentration of base.

† 'Histadyl,' Eli Lilly and Co., Indianapolis, Ind., courtesy of Dr. K. K. Chen.

‡ 'Pyribenzamine,' Ciba and Co., Summit, N. J., courtesy of Dr. Frederick F. Yonkman.

Action on blood pressure. The blocking effect on the vasodepressor action of histamine was determined in dogs. The test animals were anesthetized with sodium barbital and carotid blood pressure was recorded kymographically. All drugs were made up in distilled water and injected into the exposed femoral vein. An amount of histamine was injected to give a 40–60 mm. fall in blood pressure, the particular dose selected being used throughout the entire experiment. Five minutes after injection of the antihistaminic drug, histamine was again injected and the per cent antagonism determined from the difference between the falls obtained before and after drug administration. The standard dose of histamine was injected at 5 minute intervals until the initial response could be obtained in order to determine the duration of the drug effect. Results are shown in table 2. WIN 2848 and tripelennamine appear to be equally effective, no blocking action being observed until after the administration of a dose of 0.1 mgm./kgm.

Action in the bronchioles. *a. Lung perfusion.* Guinea pig lungs were

perfused by the method of Sollman and Von Oettingen as modified by Thornton (7). All injections were made into the perfusion cannula near the lungs. The modification in the rate of flow elicited by histamine alone was compared with

TABLE 2

Histamine blocking action in dogs

(Histamine acid phosphate injected intravenously to produce a fall in blood pressure of 40–60 mm Hg The per cent reduction in response after antihistaminic drug administration was determined)

DRUG	NUMBER OF EXPERIMENTS	DOSE I V	HISTAMINE ANTAGONISM	APPROXIMATE DURATION OF EFFECT
		mgm /kgm	*per cent*	*minutes*
WIN 2848*	3	0 10	22	20–35
	6	0 50	53	90
WIN 2876*	3	0 10	No effect	
	1	0 50	No effect	
	2	1 00	18	25
Tripelennamine	3	0 10	19	10–20
	4	0 50	58	60

* Dose expressed as mgm base

TABLE 3

Histamine blocking action on the isolated perfused guinea pig lung

DRUG	NUMBER OF EXPERIMENTS	DOSE*	CONTROL		EXPERIMENTAL		EFFECT
		mgm	*cc /min*	*cc /min*	*cc /min*	*cc /min*	*per cent*
WIN 2848	3	0 0043	45	17	44	42	93
	3	0 0087	50	25	47	46	96
WIN 2875	2	0 043	44	24	43	37	30
	4	0 087	52	27	52	49	12
WIN 2876	3	0 087	56	32	56	48	33
	3	0 43	49	23	49	48	96
Tripelennamine	3	0 043	49	23	43	38	19
	4	0 087	51	28	48	46	87
Epinephrine	5	0 01	53	26	52	58	121

* Dose expressed as mgm base

that obtained after injecting histamine with the antihistaminic drug. Results obtained are shown in table 3. These data suggest that WIN 2848 is more active than tripelennamine and as active as epinephrine in its bronchodilator action.

b. Histamine asthma in guinea pigs. Healthy guinea pigs, weighing from 250–

mgm./kgm. (1.64 micromols) whereas in the antihistaminic assay the dose of histamine employed is equivalent to increasing the LD_{50} to 0.5 mgm./kgm. (4.5 micromols). It might be assumed from these data that 1 micromol of WIN 2848 and tripelennamine neutralized approximately 57 and 28 micromols of histamine, respectively.

PROTECTIVE ACTION AGAINST THE PERCUTANEOUS EFFECTS OF HISTAMINE IN MAN. Histamine diphosphate, 0.5–2.0 per cent, in a vehicle containing carbitol, octyl alcohol, ethyl alcohol and distilled water was applied to the skin of the forearm. The local reactions noted were itching, erythema and wheal formation. WIN 2848 in a concentration of 2.5–10 per cent in the above vehicle was applied 10 minutes to 5 hours before histamine. This treatment resulted in a marked reduction in the severity of the symptoms. Luduena and Ananenko (12) have described these findings in more detail elsewhere.

TABLE 6

Acute toxicity of antihistaminic drugs in albino mice

DRUG	LD_{50} ± S.E. IN MGM./KGM. BASE	
	Intravenous	Subcutaneous
WIN 2848	17 ±1.0	46 ± 8
WIN 2875	20 ±2.0	142 ± 8
WIN 2876	18 ±0.6	85 ±12
Tripelennamine	17 ±1.0	64 ± 5
Diphenhydramine	31 ±0.8	126 ± 7

TOXICITY. The acute toxicity of each of these compounds, as the hydrochloride salt in aqueous solution, was determined in male albino mice weighing between 18 and 22 grams by both intravenous and subcutaneous administration. The volume of solution injected was maintained at 0.35 ± 0.15 cc., and a rate of 1.0 cc./minute was employed for the intravenous injections. The mice were kept in a controlled environment at 24.5°C. Deaths were tabulated during the 24 hours following injection after which time the LD_{50} ± s.e. was calculated (14). Results obtained are shown in table 6. The three WIN compounds appear to be of the same order of toxicity as tripelennamine when administered intravenously in mice whereas diphenhydramine is approximately one-half as toxic. By subcutaneous injection, tripelennamine and WIN 2848 appear to be equally toxic while diphenhydramine is approximately one-third as toxic. Of the two halogenated derivatives of WIN 2848, both are less toxic than the parent compound, the chloro- (WIN 2875) being less toxic than the bromo- (WIN 2876) compound by subcutaneous injection in mice.

DISCUSSION. The description by Staub (1) in 1939 of the antihistaminic action of N-phenyl-N-ethyl-N′N′-diethylethylenediamine (1571F) initiated a series of investigations in an effort to determine the importance of the various substituents on the nucleus $=NCH_2CH_2N=$. It was soon established that two methyl groups on one of the nitrogens were favorable for antihistaminic action (Hal-

pern, 2) Similarly the substituents on the second amine have been varied The substitution of a benzyl ('Anteigan') in place of the ethyl group of N'-phenyl-N'-ethyl N, N-dimethylethylenediamine (2325 RP), the replacement of the phenyl group of the latter by a 2 pyridyl (tripelennamine), a 2 thenyl (thenylpyramine) or 3-thenyl (WIN 2848) group has resulted in a progressive increase in antihistaminic activity The observed increases would not be anticipated from these changes in structure This is well illustrated by the observed difference in activity between the 2 thenyl (log dose 7 7) and 3 thenyl (log dose 8 3) analogs Litchfield *et al* (15) have reported that halogenation of the 2 thenyl derivative resulted in a distinct increase in antihistaminic activity Our halogenated derivatives (WIN 2875 and WIN 2876) were found to be significantly less active than the unsubstituted compound (WIN 2848) This difference between the 2- and 3-thenyl analogs may arise from the difference in the position of the halogen groups In the former they are in the 5 and in the latter in the 2 position on the thiophene ring

Feinberg (13) in his recent review of antihistaminic agents has indicated a reasonably good correlation between antihistaminic action in experimental animals and subsequent clinical experience in the treatment of allergic disorders in man The data presented here suggest that WIN 2848 may also be found to be a useful agent for the treatment of allergies

SUMMARY

1 WIN 2848 is a highly active antagonist of histamine and is more potent in its antihistaminic actions than are the chloro- and bromo- substituted analogs, WIN 2875 and WIN 2876

2 The antihistaminic drugs investigated here stimulate the isolated guinea pig uterus

3 WIN 2848, in a dose of 0 013 mgm /kgm , protects guinea pigs against 2 8 intravenous lethal doses of histamine

4 WIN 2848 diminishes the effects of histamine given percutaneously to man

5 WIN 2848 does not appear to be significantly more toxic than structurally similar antihistaminic drugs

The authors wish to acknowledge the technical assistance of Miss Estelle Ananenko, Mrs Gwendolyn Jones, Mrs Evelyn Alexander, Mr D K Seppelin and Mrs Nettie Beglin

REFERENCES

1 Staub, A M Ann Inst Pasteur, 63 400 and 485, 1939
2 Halpern, B N Arch internat de pharmacodyn et de therap , 68 339, 1942
3 Bovet, D , Horclosis, R , and Walthert, F Compt rend Soc de Biol , 138 99, 1944
4 Mayer, R L Huttrer, C P , and Scholz, C R Science, 102 93, 1945
5 Lee, H M , Dinwiddie, W G , and Chen, K K This Journal, 90 83, 1947
6 Miller, L C , Becker, T J , and Tainter, M L This Journal, 92 260, 1948
7 Thornton, J W Quart J Exper Physiol , 21 305, 1932
8 Siegmund, O H , Granger, H R , and Lands, A M This Journal, 90 254, 1947

mgm./kgm. (1.64 micromols) whereas in the antihistaminic assay the dose of histamine employed is equivalent to increasing the LD_{50} to 0.5 mgm./kgm. (4.5 micromols). It might be assumed from these data that 1 micromol of WIN 2848 and tripelennamine neutralized approximately 57 and 28 micromols of histamine, respectively.

PROTECTIVE ACTION AGAINST THE PERCUTANEOUS EFFECTS OF HISTAMINE IN MAN. Histamine diphosphate, 0.5–2.0 per cent, in a vehicle containing carbitol, octyl alcohol, ethyl alcohol and distilled water was applied to the skin of the forearm. The local reactions noted were itching, erythema and wheal formation. WIN 2848 in a concentration of 2.5–10 per cent in the above vehicle was applied 10 minutes to 5 hours before histamine. This treatment resulted in a marked reduction in the severity of the symptoms. Luduena and Ananenko (12) have described these findings in more detail elsewhere.

TABLE 6

Acute toxicity of antihistaminic drugs in albino mice

DRUG	LD_{50} ± S.E. IN MGM./KGM. BASE	
	Intravenous	Subcutaneous
WIN 2848	17 ±1.0	46 ± 8
WIN 2875	20 ±2.0	142 ± 8
WIN 2876	18 ±0.6	85 ±12
Tripelennamine	17 ±1.0	64 ± 5
Diphenhydramine	31 ±0.8	126 ± 7

TOXICITY. The acute toxicity of each of these compounds, as the hydrochloride salt in aqueous solution, was determined in male albino mice weighing between 18 and 22 grams by both intravenous and subcutaneous administration. The volume of solution injected was maintained at 0.35 ± 0.15 cc., and a rate of 1.0 cc./minute was employed for the intravenous injections. The mice were kept in a controlled environment at 24.5°C. Deaths were tabulated during the 24 hours following injection after which time the LD_{50} ± s.e. was calculated (14). Results obtained are shown in table 6. The three WIN compounds appear to be of the same order of toxicity as tripelennamine when administered intravenously in mice whereas diphenhydramine is approximately one-half as toxic. By subcutaneous injection, tripelennamine and WIN 2848 appear to be equally toxic while diphenhydramine is approximately one-third as toxic. Of the two halogenated derivatives of WIN 2848, both are less toxic than the parent compound, the chloro- (WIN 2875) being less toxic than the bromo- (WIN 2876) compound by subcutaneous injection in mice.

DISCUSSION. The description by Staub (1) in 1939 of the antihistaminic action of N-phenyl-N-ethyl-N′N′-diethylethylenediamine (1571F) initiated a series of investigations in an effort to determine the importance of the various substituents on the nucleus $=NCH_2CH_2N=$. It was soon established that two methyl groups on one of the nitrogens were favorable for antihistaminic action (Hal-

A SIMPLE ASSAY FOR PARASYMPATHOLYTIC AGENTS USING THE LACRIMATION RESPONSE IN RATS

MARTIN M WINBURY, DOROTHY M SCHMALGEMEIER, AND W E HAMBOURGER

With the technical assistance of Doris A Jonsson

Pharmacology Laboratories, Research Department, G D Searle and Co, Chicago

Received for publication September 20, 1948

There is need for a simple *in vivo* assay for the study of parasympatholytic agents useful for routine testing Bülbring and Dawes recently described a graded response assay based on the inhibition of salivary secretion in the anesthetized cat (1) This method is excellent but does not lend itself readily to the study of parasympathomimetic inhibition following administration by various routes, nor to the determination of duration of action To satisfy these needs, an assay for parasympatholytic activity has been developed which can be carried out routinely on unanesthetized rats It makes use of an all-or none response and permits simple statistical analysis The inhibition of the response induced by a parasympathomimetic agent is used as the end point in the assay The use of this method for the study of three compounds is described below

PROCEDURE Acetyl β methylcholine chloride (Mecholyl) is used as the parasympathomimetic stimulant throughout this assay (2) At a dose level of 10 mgm /kgm administered intaperitoneally to normal rats, the most pronounced and consistent response is a profuse lacrimation slightly tinged with blood The inhibition of this response is used as the end point of the assay The rat is the animal of choice because the response is always clean cut and easily detected in this species Lacrimation occurs about 2 minutes after injection of Mecholyl and usually continues for 5 minutes, all evidence of lacrimation disappears within 15 minutes, and the animals can be used again for duration studies

In general, the experimental design is the same as that for other all or none assays Potency determination is based on comparison of the doses of unknown and standard parasympatholytic agent which protect 50 per cent (PD_{50}) of the rats against lacrimation due to 10 mgm /kgm of Mecholyl injected intaperitoneally Four groups of 6 to 12 rats each, selected at random from the same "colony," are given logarithmically graded doses of the unknown or standard by the intraperitoneal route Fifteen minutes after administration of the parasympatholytic agent all animals are injected with Mecholyl, and the number of animals from each group responding is noted These data are used for estimating the PD_{50} and relative potency values compared to a standard agent By repeating the Mecholyl stimulation at 15 or 30 minute intervals, the duration of action at the various dose levels of the parasympatholytic can be studied If the duration is to be followed for a long period of time (i e, after oral administration) it is advisable to reduce the frequency of Mecholyl stimulation When the test compound is given by stomach tube, the animals are injected intraperitoneally with Mecholyl 30, 60, and 90 minutes after the oral dosage It has been found that withholding food from the animals for 16 hours prior to the test decreases the variability of the results

Statistical analysis of the all or none assay is well established (3, 4, 5), and the choice of methods depends upon the degree of accuracy desired

RESULTS AND DISCUSSION. The dose of Mecholyl (10 mgm./kgm.) used to induce lacrimation is well above the ED_{100} (table 1) and always induces lacrimation in normal untreated animals. In order to justify the frequent injections of Mecholyl in the duration studies, it was necessary to establish that previous doses of Mecholyl do not affect the sensitivity to subsequent doses of this drug. Toward this end the intraperitoneal ED_{50} for lacrimation was determined in 3 groups of rats equally divided between the sexes, as follows: (1) untreated control animals; (2) Mecholyl, 10 mgm./kgm. intraperitoneally, 15 minutes prior to assay; and (3) Mecholyl, 10 mgm./kgm., both at 15 and 30 minutes prior to the ED_{50} determination. Table I summarizes the results of this experiment as calculated by the method of Wilcoxon and Litchfield (5). There is no significance of difference (P = 0.05) for the ED_{50} and slope values of the 3 groups, indicating that 10 mgm./kgm. of Mecholyl is not cumulative and does not influence the response to

TABLE I

Data showing effect of pretreatment with Mecholyl on subsequent ED_{50} of Mecholyl for lacrimation in unanesthetized rats

GROUPS	NO. OF ANIMALS	ED_{50}	$f_{ED_{50}}$*	SLOPE	f_{slope}*
		mgm./kgm.			
Untreated controls	48	0.52	1.31	1.81	1.38
Mecholyl 10 mgm./kgm. I. P. 15 min. before assay	48	0.56	1.35	1.70	1.48
Mecholyl 10 mgm./kgm. I. P. 15 and 30 min. before assay	48	0.55	1.31	1.95	1.53
Combined male results	72	0.54	1.20	1.62	1.18
Combined female results	72	0.54	1.21	1.86	1.36

* The "f" of a term is the factor by which the term is multiplied and divided to determine its confidence limits at P = 0.05.

subsequent injections. Likewise the data for each sex, when combined, indicate no sex variation in response (table I).

Previously in this laboratory compound SC 1703[1] (Diethylaminoethyl-xanthene-9-carboxylate methochloride) was found to have outstanding parasympatholytic activity. It was, therefore, arbitrarily selected as the standard for comparison in preference to atropine. The results of 14 assays of SC 1703 are given in table II, lines 1–14. These data were obtained over the course of 6 months, employing 3 different colonies of 100 rats each. The colonies were replaced every few months when the rats became too large to handle with ease. None of the animals was used more than once a week, and groups for assay were selected at random from the available animals. For the purpose of comparison, the results for each colony have been combined and the values in table II, lines 15–17, determined in the usual manner (5). It will be noted that the PD_{50} and slope values are quite consistent within each colony and among the colonies (P = 0.05). Weight, age and sex differences have no apparent influence. These facts tend to

[1] SC 1703 was synthesized by Richard A. Robinson and SC 1222 by John W. Cusic, to whom the authors wish to express their gratitude.

indicate that it is not necessary to assay the unknown and standard parasympatholytic agents on the same group of animals and that random selection is per-

TABLE II

Data showing results of 14 assays on the parasympatholytic activity of SC 1703 against lacrimation due to 10 mgm./kgm. of Mecholyl in the unanesthetized rat

LINE	COLONY AND SEX (WEIGHT RANGE)	NO OF ANIMALS	15 MINUTES				30 MINUTES			
			PD_{50}	$f_{PD_{50}}$*	Slope	f_{slope}*	PD_{50}	$f_{PD_{50}}$*	Slope	f_{slope}*
			mgm / kgm				*mgm / kgm*			
1	I ♀ 270–310	20	0 87	1 99	1.71	1 51				
2	I ♀ 300–340	24	0 95	1 98	1 83	1.84	1 60	1 37	1 48	1.18
3	I ♀ 300–340	24	1 03	1 97	1 81	1 65				
4	I ♀ 300–350	24	0 70	1 93	2 79	2 82	1 20	1 60	1 80	1.47
5	I ♀ 300–350	24	1 00	1 70	2 25	1 80	2 00	1 84	1 71	1 59
6	II ♀ 180–210	48	0 86	1 51	2 58	2 10				
7	II ♀ 200–240	24	0 94	1 62	1 85	1 51	1 28	1 55	1 74	1 38
8	II ♀ 220–280	24	0 72	1 70	1 94	1 63	1 15	1 68	1 91	1 58
9	II ♀ 220–280	24	0 73	1 81	2 12	1 90	1 27	1 58	1 77	1 45
10	III ♂ 160–200	24	0 94	1 91	1 85	1 85	1 41	1 47	1 62	1 30
11	III ♂ 160–200	24	0 92	1 65	1 88	1 58	1 42	1.60	1 82	1 50
12	III ♂ 160–200	24	0 89	1 68	1 93	1 63	1 42	1 60	1 82	1 50
13	III ♂ 160–200	24	1 03	1 60	2 02	1 58	1 55	1 56	1 74	1 42
14	III ♂ 210–320	48	0 62	1 48	2 40	1 65	1 48	1 51	2 06	1 56
15	Combined ♀ Colony I	116 (72)†	0 86	1 25	1 84	1 22	1 60	1 28	1 73	1 23
16	Combined ♀ Colony II	120 (72)†	0 75	1 34	2 28	1 42	1 13	1 39	1 67	1 27
17	Combined ♂ Colony III	144 (144)†	0 84	1 24	1 80	1 14	1 46	1 23	1 78	1 13

* Same as in Table I

† In each entry the upper number is for 15 minutes and figure in parentheses is for 30 min

missible. As a further proof of this, the results of an experiment in which four groups of rats were assayed with the standard on the same day are given in table II, lines 10–13. Again no significant difference in the slope and PD_{50} appear.

TABLE III

Comparison of the parasympatholytic activities of SC 1703 with atropine sulfate and SC 1222 in the unanesthetized rat (intraperitoneal administration)

1703 (9-2-48)*		Atropine sulfate (9-2-48)*		1703 (4-29-48)*		1222 (4-29-48)*	
Dose	P/D†	Dose	P/D†	Dose	P/D†	Dose	P/D†
mgm./kgm.		*mgm./kgm.*		*mgm./kgm.*		*mgm./kgm.*	
0.25	0/12	0.125	0/12	0.5	3/12	0.5	0/12
0.5	7/12	0.25	1/12	1.0	7/12	1.0	1/12
1.0	9/12	0.50	9/12	2.0	11/12	2.0	3/12
2.0	10/12			4.0	10/12	4.0	7/12

	1703 (9-2-48)	Atropine sulfate	1703 (4-29-48)	1222
PD_{50}	0.62	0.38	0.86	3.38
$f_{PD_{50}}$‡	1.48	1.28	1.51	1.60
Slope	2.40	1.54	2.58	2.32
f_{slope}‡	1.65	1.23	2.10	1.83
Potency	1	1.63	1	0.25
$f_{Potency}$‡	—	1.60	—	1.83

* The date of the assay is given in parentheses.

† $\frac{P}{D} = \frac{\text{Number protected}}{\text{Number dosed}}$.

‡ Same as in Table I.

TABLE IV

Comparison of the parasympatholytic activity of SC 1703 following intraperitoneal and oral administration

Intraperitoneal		Oral	
Dose	P/D†	Dose	P/D†
mgm./kgm.		*mgm./kgm.*	
0.5	0/6	32	1/5
1.0	3/6	64	2/5
2.0	4/6	128	3/5
4.0	6/6	256	5/5

	Intraperitoneal	Oral
PD_{50}	1.03	84
$f_{PD_{50}}$*	1.60	1.92
Slope	2.02	2.15
f_{slope}*	1.58	2.01
Ratio	1	82
f_{Ratio}*	—	2.23

* Same as in table I.

† Same as in table III.

The results at 15 minutes have been used for potency estimation since this is the point of maximum inhibition.

As an example of the use of this method, a comparison of the intraperitoneal parasympatholytic activity of the standard (SC 1703) with atropine sulfate, and

compound SC 1222[1] (9-Diethylaminoethylphenothiazine methoiodide) is presented in table III. The data show that atropine and SC 1222 have, respectively, 163 and 25 per cent of the activity of SC 1703, and these differences are significant.

The use of the method for determining the oral activity of SC 1703 is illustrated in table IV. The results at 90 minutes after oral administration were used because this was the point of maximum protection. The usual results at 15 minutes for intraperitoneal administration are also given. On the basis of these data, the oral PD_{50} is approximately 82 times the intraperitoneal PD_{50}.

SUMMARY

A simple all-or-none assay for parasympatholytic agents using unanesthetized rats is described. The assay is based on the inhibition of lacrimation induced by Mecholyl Weight, age and sex differences have no apparent influence on the results Action following various routes of administration can be studied. Examples are given for intraperitoneal and oral routes of administration.

REFERENCES

1 BÜLBRING, E , AND DAWES, G S THIS JOURNAL, 84: 177, 1945
2 GOODMAN, L , AND GILMAN, A The Pharmacological Basis of Therapeutics, The Macmillan Co , New York, pg 360, 1941
3 BLISS, C I Quart J Pharm and Pharmacol , 11: 192, 1938
4 MILLER, L C , AND TAINTER, M L Proc Soc Exper Biol & Med , 57: 261, 1944.
5 WILCOXON, F , AND LITCHFIELD, J T , JR Authors reprint, privately printed, of paper presented at Federation Meetings (Litchfield and Wilcoxon Fed Proc , 7: 240, 1948)

THE EFFECT OF 3-ACETYLPYRIDINE AND NIACINAMIDE ON THE PERFUSED HEART

K. BRAUN[1]

Cardiovascular Department, Medical Research Institute, Michael Reese Hospital, Chicago, Illinois[2]

Received for publication September 24, 1948

The recent development of structural analogues of vitamins has made possible new approaches to the problems of vitamin deficiency, since some of these compounds have been shown to be biological antagonists for the related vitamins. They exhibit a specific inhibition of growth of those bacteria for which the vitamin is an essential nutritive element. In animals the administration of these substances produces manifestations which have been associated with the deficiency of the particular vitamins, and these effects are reversed or prevented by the administration of the vitamins. The best known example of this phenomenon is the competitive inhibition between sufonamides and para-aminobenzoic acid (1). Woolley (2) has made a series of experimental observations with compounds related to various vitamins and his work has done much to develop the concept of biological antagonism. He found that two compounds showed marked antagonism against niacinamide (3); pyridine-3-sulfonic acid inhibited microbial growth, while 3-acetylpyridine produced signs of deficiency in animals which did not appear when niacin also was administered.

Rachmilewitz and Braun (4) have shown in a series of clinical investigations, that niacin deficiency may cause marked alterations in the electrocardiogram. They described abnormalities of the T wave, S-T segment, and even the appearance of intraventricular block. The prompt disappearance of these changes following niacin therapy led to the conclusion that these electrocardiographic abnormalities are caused by niacin deficiency and hence are a part of the pellagra syndrome.

In order to determine whether the induction of an acute "deficiency" state in the heart by the administration of 3-acetylpyridine could be demonstrated, we have undertaken studies on the isolated rabbit heart. 3-Acetylpyridine, an anologue of niacin in which the —COOH group of the vitamin had been exchanged for —$COCH_3$, was chosen because it has produced signs of pellagra in experimental animals.

METHODS. A modified Langendorff preparation of the rabbit's heart was made. The perfusion fluid entered the aorta above the valves; a small hole was made in the wall of the left ventricle, through which the fluid left the heart. A modified Tyrode-Locke solution was used as perfusion fluid; each liter contained: sodium chloride 9.0 grams, sodium bicarbonate 1.0 gram, potassium chloride 0.2 gram, anhydrous calcium chloride 0.2 gram, glucose 1.0 gram, magnesium chloride 0.1 gram. A mixture of 5 per cent carbon dioxide and 95 per cent oxygen was bubbled continuously through this solution. The pH of the solution was

---

[1] Hadassah Fellow from Jerusalem. Aided by the Emil and Fanny Wedeles Fund.

[2] The department is supported in part by the Michael Reese Research Foundation.

7.5 and it remained constant, even when 3-acetylpyridine (in a concentration of 1:500) or niacinamide (in a concentration of 1:1000) were added. The perfusion apparatus was connected with two containers so that two different solutions could be interchanged quickly. The temperature of the perfusion fluid was kept constant at 37°C., by means of two water-jackets. A small surgical needle with a thread soaked in saline was inserted carefully into the epicardium at the apex of the left ventricle. This thread was in constant touch with the effluent, which was collected in a small container. The thread was used as the direct electrode. Serial electrocardiograms were taken on a direct writing machine (Viso-cardiette).

Twenty-eight experiments were performed according to the method described. Several concentrations of 3-acetylpyridine and of niacinamide were used in these experiments.

Results. 1. *Controls.* Five control experiments were performed without addition of any drug, in order to observe the reliability of the method. It was found that during a period of 90 minutes the heart rate did not change significantly. The T-waves showed a gradual decrease in height during this time. After 90 minutes, the heart rate became progressively slower and auriculo-ventricular block of various degrees developed. In two of these experiments premature beats were seen at the beginning, but these disappeared quickly after the heart had been perfused.

2. *Administration of 3-acetylpyridine.* 3-Acetylpyridine was given in two ways: a) in a less concentrated form mixed with the perfusion fluid, and b) in a more concentrated form administered by a syringe directly into the cannula in the heart, while the perfusion fluid was flowing from the container.

A concentration of 1:1000 to 1:5000 of the drug was used as a continuous perfusion in 9 experiments. The drug was administered only after the electrocardiogram was unchanged for a period of at least 15 minutes. In all these experiments, disturbances of rhythm appeared within 10 to 34 minutes after the beginning of the infusion of the antivitamin (table 1). In seven, A–V block of second or third degree was seen and in two, ventricular fibrillation occurred. The A–V block was reversible in 8 to 15 minutes by a perfusion with niacinamide in concentrations of 1:100,000 to 1:500,000. In 3 of these experiments, the entire procedure could be repeated a second time. A protocol of a typical experiment is shown in table 2. In one experiment, the A-V block could not be reversed by niacinamide. Ventricular fibrillation occurring after administration of the antivitamin was abolished, in 1 of the 2 experiments, by perfusion with niacinamide.

In 3 experiments, electrocardiographic abnormalities were produced with the antivitamin and the effect of continued perfusion with normal fluid alone was observed. It was found that such a control perfusion for a period of 25 minutes did not abolish the arrhythmias. In four experiments, 3-acetylpyridine was given in a more concentrated form (1:500 to 1:1000) by a syringe directly into the perfused heart. Arrhythmias appeared in every instance after 1 to 3 minutes. This effect could be reversed by niacinamide after 1 to 4 minutes, administered in the same way and in concentration of 1:50,000 and 1:100,000. In one of these experiments the procedure was repeated 5 times and each time the arrhythmia was abolished by niacinamide.

The heart was perfused from the beginning in 3 experiments with niacinamide in a concentration of 1:100,000. The direct administration of 3-acetylpyridine

in a concentration of 1:1000 by a syringe, while the perfusion fluid was running, did not produce any abnormalities.

3. *Administration of niacinamide.* In 3 experiments, arrhythmias of various kinds were observed from the beginning and these did not change for a period of

TABLE I

Effects of continuous perfusion with 3-acetylpyridine and niacinamide

EXP. NO.	EFFECT OF ANALOGUE	TIME REQUIRED	EFFECT OF NIACINAMIDE	TIME REQUIRED
		min.		*min.*
1	A-V block	18	Reversal to normal	12
2	A-V block	10	Reversal to normal	14
3	A-V block	26	Reversal to normal	12
4	A-V block	23	No change	25
5	Ventricular fibrillation	21	Reversal to normal	8
6	A-V block	16	Reversal to normal	8
7	A-V block	34	Reversal to normal	15
8	Ventricular fibrillation	16	No change	15
9	A-V block	25	Reversal to normal	11

TABLE II

Summary of data of one experiment (No. 6)

TIME AFTER START OF EXPERIMENT	DRUG PERFUSED	CONCENTRATION	ECG APPEARANCE
min.			
15	0 (control)	0	Normal
20	3-Acetylpyridine	1:5000	Normal
24	3-Acetylpyridine	1:5000	Normal
28	3-Acetylpyridine	1:5000	Normal
32	3-Acetylpyridine	1:5000	Normal
36	3-Acetylpyridine	1:5000	A-V block (second degree)
40	3-Acetylpyridine	1:5000	A-V block (second degree)
44	Niacinamide	1:500,000	A-V block (second degree)
48	Niacinamide	1:500,000	A-V block (second degree)
52	Niacinamide	1:500,000	Normal
56	Niacinamide	1:500,000	Normal
60	Niacinamide	1:500,000	Normal
64	3-Acetylpyridine	1:5000	Normal
68	3-Acetylpyridine	1:5000	Normal
72	3-Acetylpyridine	1:5000	Normal
76	3-Acetylpyridine	1:5000	A-V block (second degree)
80	3-Acetylpyridine	1:5000	A-V block (second degree)
84	Niacinamide	1:500,000	A-V block (second degree)
88	Niacinamide	1:500,000	A-V block (second degree)
92	Niacinamide	1:500,000	Normal
96	Niacinamide	1:500,000	Normal

30 minutes. The addition of niacinamide to the perfusion fluid in a concentration of 1:200,000 and 1:500,000 abolished these disturbances in 2 cases after 8 and 11 minutes. Thus the beneficial effect of niacin on the isolated heart preparation, observed by Calder (5), was confirmed.

DISCUSSION It is reasonable to assume that the administration of 3 acetylpyridine caused an acute interference with the normal functions of niacinamide in the isolated heart as has been shown to be the case in intact animals In this way an acute "deficiency" apparently has been produced, which causes marked alterations in the function of the heart, as demonstrated by the appearance of various arrhythmias This may be regarded as additional evidence for our previous observations (4) that electrocardiographic abnormalities may occur as a sign in niacin deficiency

Niacinamide plays an important part in the normal metabolism of the animal It is a constituent of two important compounds which are involved in many enzymatic reactions diphosphopyridine nucleotide (coenzyme I) and triphosphopyridine nucleotide (coenzyme II) Niacinamide is therefore probably necessary for the normal activity of the heart and its deficiency should interfere with the function of the heart

In the isolated heart there is a continuous perfusion of the heart muscle and it is possible that some niacinamide may be washed out by the fluid, especially since a continuous exchange between niacinamide and the coenzymes takes place Thus, in the course of experiments which involve perfusion of an isolated heart with Ringer's solution, a relative "deficiency" may develop and impair cardiac function Addition of niacinamide and perhaps of other vitamins to such perfusion fluids is suggested since they may exert a beneficial effect on the perfused heart and permit better standardization of this preparation

Generally, larger amounts of the antagonist are needed to reverse the action of the normal metabolite In our experiments it was found that the concentration of the antivitamin had to be at least 100 times greater than that of the vitamin to cause definite changes This suggests that the normal vitamin has a much greater affinity for the affected system than does the antagonist The reversal of the change in the series with continuous perfusion also took less time (average, 13 minutes) than did the production of the electrocardiographic abnormalities (average, 21 minutes)

SUMMARY

The perfusion of an isolated heart with an analogue of niacin, 3 acetylpyridine produced marked electrocardiographic abnormalities Administration of niacinamide caused a reversal of these changes

I am indebted to Dr L N Katz for his advice in the execution of this study and to Dr S Rodbard for his suggestions in the preparation of this report

REFERENCES

1 WOODS, D D Brit J Exper Path, 21 74, 1940
2 WOOLLEY, D W Physiol Rev, 27 308, 1947
3 WOOLLEY, D W J Biol Chem, 157. 435, 1945
4 a RACHMILEWITZ, M, AND BRAUN, K Brit Heart J, 7 72, 1945
b BRAUN, K, ROTH, J, AND SUESSKIND, S J Ped, 30 177, 1947
c RACHMILEWITZ, M, AND BRAUN, K Am Heart J, 36 248, 1948
5 CALDER, R M Proc Soc Exper Biol and Med, 65 76, 1947
6 KODICEK, E, CARPENTER, K J, AND HARRIS, L J Lancet, 2 491, 1946

THE EFFECT OF ETHANOL AND VARIOUS METABOLITES ON FLUOROACETATE POISONING[1]

JOHN O. HUTCHENS, HAROLD WAGNER, BETTY PODOLSKY, AND T. M. McMAHON

Toxicity Laboratory and the Department of Physiology, University of Chicago, Chicago Illinois

Received for publication September 27, 1948

INTRODUCTION. The need for a method of treating animals or men poisoned with sodium mono-fluoroacetate[2] (1080) has been evident for some time. Rodent control operations in which 1080 was used have resulted in accidental poisoning of sheep and to a minor extent of cattle and swine.[3] The possibility that pets will be accidentally poisoned exists with all rodenticides but is particularly strong with 1080. The marked susceptibility of dogs to fluoroacetate (1) and the fact that they may eat rodents poisoned with 1080 makes secondary poisoning of this species possible even when effective measures are taken to deny them access to poisoned baits or water.

Previous attempts to treat 1080 poisoning were based on the following:

(a) The concept that fluoroacetate specifically and competitively inhibits acetate metabolism (2, 3). It was found (4) that acetate administered in large amounts either before or after fluoroacetate failed to prevent death or prolong survival in rabbits. (However, in the isolated rabbit heart, perfusion with acetate was found to protect against the action of fluoroacetate (4)).

(b) The fact that cardiac arrythmias and ventricular fibrillation are the cause of death in some species (1). Quinidine, procaine, and p-aminobenzoic acid have been employed to prevent or control these in monkeys, the results being encouraging but not strikingly successful (4).

(c) The fact that fluoroacetate produces tonic convulsions leading to death from respiratory failure in some species (1). Pentobarbital anesthesia was reported to be of no value in dogs (1).

(d) The belief that fluoroacetate inhibits intermediary carbohydrate metabolism at a step critical for resynthesis of phosphocreatine (5). Cori, Colowick, et al. administered citrate, glutamate, and succinate to rats poisoned with fluoroacetate without therapeutic effect (5).

The possibility of a new and successful attack on the problem of treating 1080 poisoning was raised by recent experiments on the biochemical mechanism of inhibition of oxidations by fluoroacetate. Hutchens and McMahon (6) reported that in unicellular organisms 1080 is not a specific inhibitor of acetate metabolism

[1] The work described in this paper was done under contract with the Medical Division, Chemical Corps, U. S. Army and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

[2] For the origin of the symbol 1080 see Kalmbach, F.: Science, **102**: 232, 1945.

[3] Personal communication, Mr. Justus Ward, U. S. Fish and Wildlife Service.

and that acetate oxidation is, as a matter of fact, less sensitive to inhibition by 1080 than is pyruvate oxidation Black and Hutchens (7) have shown that, if acetate is added to yeast suspensions after 1080, a prolonged induction period precedes oxidation of the acetate They showed, however, that ethanol in catalytic amounts markedly shortens this induction period More extensive experiments along these same lines have been carried out in this laboratory and will be reported elsewhere

We therefore undertook a study of the effects of ethanol, acetate, and various other substances on 1080 poisoned animals The results, which are presented below, indicate that 1080 poisoning can be successfully combatted in some species of animals

MATERIALS AND METHODS *Animals* Mice used were Maple Grove male and female albinos ranging in weight from 20 to 27 grams No differences in susceptibility of the two sexes were noted, and no distinction is made between them in reporting results Before use, and following the treatment and early observation period, the mice were kept in air conditioned animal quarters at 27°C Laboratory temperatures in the interim periods ranged in various experiments from 18 to 25°C Treated and control groups of mice in any experiment were exposed to identical conditions

Guinea pigs used ranged in weight from 175 to 900 grams, mostly about 300 grams

Rabbits weighed from 2 to 3 kgm

Dogs ranged in weight from 4 to 12 kgm and were purchased from dealers All dogs received homologous serum for respiratory infections and distemper vaccine at least two weeks before use The quarters in which guinea pigs, rabbits and dogs were housed varied in temperature from 20 to 30°C in various experiments

Mice and dogs were fed Wayne Dog Blox, guinea pigs and rabbits Purina Rabbit Pellets While the animals had food and water available at all times, none except successfully treated ones ate or drank appreciable amounts during the first twenty four hours after poisoning

Poisoning The desired dose of 1080 dissolved in normal saline was administered by the following routes and in volumes indicated mice, 10 cc /kgm subcutaneously, guinea pigs, 1 0 cc /kgm intraperitoneally, rabbits, 0 5 cc /kgm subcutaneously, dogs, 0 1 cc /kgm intravenously

Mortality data Animals were closely observed for at least the first three hours after poisoning Times of death were recorded so that average survival time could be calculated Deaths occurring within ten days were recorded Wherever sufficient data were available, the LD_{50}, its standard error, and the fiducial limits ($p = 0\ 01$) were calculated by the method of Bliss (8) As will be noted below, we have employed a graphical test of significance of differences in percentage mortality Points falling outside the curve delimiting the fiducial limit for $p = 0\ 01$ were considered as significantly different

EXPERIMENTAL RESULTS *Description of poisoned animals* In general our observations on 1080 poisoned animals confirm those previously reported (1) for animals poisoned with methyl fluoroacetate Certain additional observations are perhaps worthy of mention

Mice poisoned with 1080 exhibited no signs of poisoning for thirty to forty-five minutes except with the highest doses employed At these doses (40–100 mgm /kgm) signs were evident in twenty minutes First noted in all cases was depression of the animals They moved little, but scratching the cage or touching the animals caused them to start and frequently to move about By forty-

five to sixty minutes the mice were prostrate and convulsing intermittently. It was noted that both treated and untreated mice were anuric for long periods of time in spite of relatively large amounts of fluid administered.

All 1080 poisoned mice and guinea pigs at some time exhibited a hyperirritable state, responding to a stimulus by a start similar to that seen in strychnine poisoned animals. Handling of the animals in such a state sometimes produced

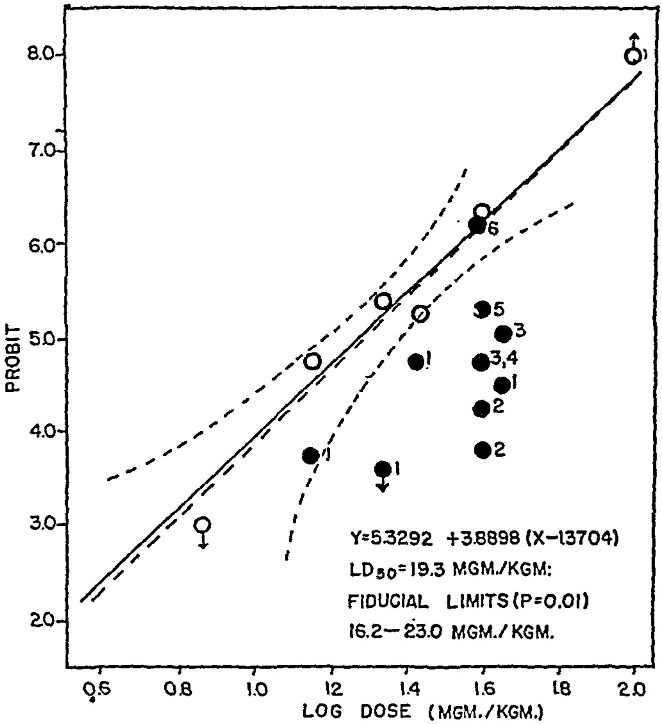


FIG. 1. DOSAGE-MORTALITY CURVE FOR MICE INJECTED SUBCUTANEOUSLY WITH 1080 AND THE EFFECT OF VARIOUS MATERIALS ON MORTALITY

Dotted curved lines indicate fiducial limits (p = 0.01). Symbols: O, untreated; ● 1, 0.5 cc. solution containing 1.3 per cent ethanol plus 13 per cent sodium acetate thirty minutes after 1080; ● 2, 800 mgm./kgm. ethanol five to ten minutes after 1080; ● 3, 2000 mgm./kgm. ethanol twenty minutes after 1080; ● 4, 4000 mgm./kgm. ethanol twenty minutes after 1080; ● 5, 800 mgm./kgm. ethanol thirty minutes, four hours, eight hours and ten hours after 1080 (repeated doses); and ● 6, mice anesthetized with 100 mgm./kgm. pentobarbital before poisoning, 2000 mgm./kgm. ethanol twenty minutes after 1080. All agents given subcutaneously in normal saline.

a convulsive seizure terminating in death. This was particularly true for guinea pigs. This suggested the use of barbiturates (*vide infra*) which, however, had no demonstrable additional effect on mortality in mice or guinea pigs when used in conjunction with ethanol.

Dogs displayed no signs of poisoning for two to four hours following the doses employed. The first sign of poisoning was an apprehensive period during which the dog walked about, then barked or howled. This was followed by one or more convulsive seizures until one of these led to death from respiratory failure.

Except in the experiments in which pentobarbital was used no dog which had a convulsive seizure survived.

Toxicity of 1080 for mice. As a basis for testing the effect of various materials on poisoning with given doses of 1080 a dosage-mortality curve for this substance was established for mice. The results obtained using a total of 77 mice at 6 different doses are shown graphically in fig. 1.

The LD_{50} for mice was found to be 19.3 mgm./kgm. with fiducial limits (p = 0.01) of 15.8–23.7 mgm./kgm.

Treatment of mice with acetate plus ethanol. Our first attempts to treat 1080 poisoning were suggested by the finding (7) that ethanol catalyzed the early oxidation of acetate by yeast previously poisoned with 1080. We, therefore, injected ethanol and acetate subcutaneously into 1080 poisoned mice using approximately the same proportions (1:10) used in yeast experiments. Relatively large amounts of the mixture were given with the idea of supporting a large fraction of the metabolism of the animals for a considerable period. The doses employed and the observed mortalities are presented graphically in fig. 1. Mice were used in groups of 10 for establishing these points.

The results of these experiments indicated that the mixture of acetate plus ethanol had a significant effect on mortality following 1080 administration. The appearance of the animals in itself gave evidence of the beneficial effects, the treated animals being neither as prostrate nor as hyperirritable as the untreated ones. The treated animals also began to eat and drink sooner than the surviving untreated animals. Those animals dying in the treated groups tended to die later than the untreated animals. The numbers, however, were too small to establish a significant difference between mean times of death.

Treatment of mice with ethanol alone. Because acetate is produced by oxidation of ethanol and because of the finding (7) that such acetate is rapidly oxidized by 1080 poisoned yeast, we decided to employ ethanol alone. The results of several such experiments are also presented graphically in fig. 1. Mice in groups of 10 were used to establish the points shown. The ethanol was injected subcutaneously, 0.2–0.5 cc. of a 10 per cent solution in normal saline being given.

These experiments indicated clearly that ethanol alone was an effective therapeutic agent in mice. The increased effectiveness of the ethanol when given 5–10 minutes after poisoning, i.e. before symptoms appeared, is apparent.

Effect of various metabolites on 1080 poisoning. Because acetate is probably not oxidized as such but is transformed to an intermediate of the tricarboxylic acid cycle before oxidation, a series of other intermediates of carbohydrate breakdown were tested for their effect on 1080 poisoning. In addition two amino acids were tested.

When 500 mgm./kgm. of the following substances were given subcutaneously ten minutes before 1080 or ten minutes following 1080 no significant effect on mortality was observed: citrate, succinate, fumarate, malate, glycerol, pyruvate, glycine and glutamic acid. Neither were 5.0 mgm./kgm. of dinitrophenol of benefit although extra oxygen consumption induced in frog muscle by this substance persists following addition of fluoroacetate (5).

Effect of barbiturate anesthesia. The possibility that ethanol exerts a thera-

peutic effect through anesthetic action seemed worthy of study. We wished to investigate this possibility particularly since hyperirritability was one of the chief signs of poisoning in mice, and we felt that in some instances convulsive seizures terminating in death were precipitated by handling incidental to treatment. We therefore combined pentobarbital anesthesia and ethanol administration with the results presented in fig. 1. The mice were anesthetized with

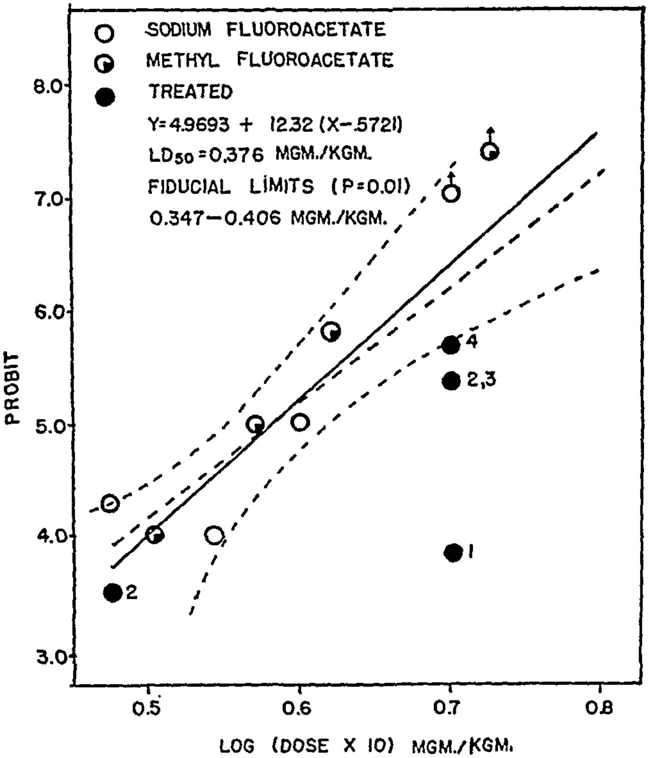


FIG. 2. DOSAGE-MORTALITY CURVE FOR GUINEA PIGS INJECTED INTRAPERITONEALLY WITH METHYL FLUOROACETATE OR 1080 AND EFFECT OF VARIOUS MEASURES ON MORTALITY

Symbols: ● 1, 800 mgm./kgm. ethanol ten minutes after 1080; ● 2, same, twenty minutes after 1080; ● 3, same, at thirty minutes, four hours, eight hours and ten hours after 1080 (repeated doses); ● 4, 800 mgm./kgm. ethanol plus 400 mgm./kgm. succinic acid twenty minutes after 1080. All agents given subcutaneously in normal saline.

pentobarbital *before* poisoning. If any conclusion is to be drawn from this experiment it must be that the pentobarbital was harmful rather than beneficial.

Experiments with guinea pigs. For testing the significance of effects of various measures on mortality among 1080 poisoned guinea pigs a dosage-mortality curve and its fiducial limits (p = 0.01) were established. The data obtained using 52 guinea pigs are presented graphically in fig. 2. Points representing the data of Chenoweth and Gilman (1) have also been plotted. To obtain these points the doses of methyl fluoroacetate used by them were converted to the equivalent doses of 1080. As might have been expected, the toxicities of the two substances are not significantly different on a molar basis.

44902

Significant effects on mortality were demonstrated only at a dose of 0.5 mgm./kgm. of 1080 (fig. 2). Most striking was the effect of 800 mgm./kgm. of ethanol injected subcutaneously as a 10 per cent solution ten minutes following this dose. When twenty minutes elapsed before administering this amount of ethanol the decrease in mortality was significant but less striking.

It will be seen from fig. 2 that the addition of 400 mgm./kgm. of succinic acid to the ethanol was of no demonstrable benefit in the treatment. Repeated doses of ethanol were not additionally effective. As with mice, pentobarbital anesthesia was of no benefit.

Experiments with rabbits. The effect of subcutaneously injected ethanol on 1080 poisoning in rabbits was studied employing two different doses of 1080. The ethanol solution was injected five minutes after the 1080. The results are presented in table 1.

TABLE 1

Effect of ethanol on 1080 poisoning in rabbits

1080	ETHANOL	MORTALITY
mgm./kgm.	*mgm./kgm.*	
0.50	None	5/5
	800	6/10
0.25	None	6/10
	800	1/10

An attempt was made to test the significance of the difference in the above mortality figures without establishing an entire dosage-mortality curve for rabbits. The data of Chenoweth and Gilman (1), who used methyl fluoroacetate, were converted to the equivalent dose of 1080 and pooled with our own. A dosage-mortality curve was established using Bliss' method (8). Our data for untreated animals indicated a somewhat higher mortality for 1080, one point falling on, the other above the line denoting the upper limit expected for $p = 0.01$. In view of the results with guinea pigs (fig. 2) this may as well be attributed to differences in the rabbits used as to differences in the two forms of fluoroacetate.

In spite of this tendency of our control animals to fall at or above the upper limits of the curve, the ethanol treated groups were well outside the lower limits of the curve. There seems, therefore, no doubt as to the significance of the effect of the ethanol.

Experiments on dogs. Analysis of data previously obtained in this laboratory (9) and of those presented by Chenoweth and Gilman (1) indicated that a dose of 0.1 mgm./kgm. of 1080 given intravenously to dogs should result in mortality of about 75 per cent. When ten dogs were given this dose and 800 mgm./kgm. of ethanol were given simultaneously (both materials injected intravenously), seven of the ten died. Since dogs given this amount of ethanol showed no symptoms of 1080 poisoning for four to six hours (av. 4.1), we studied the blood

ethanol concentrations following 800 mgm./kgm. of ethanol given intravenously. In a representative experiment the following values in mgm. ethanol/cc. of blood were found: 30 min., 0.93; 90 min., 0.72; 135 min., 0.58; 180 min., 0.47; 225 min., 0.35; 270 min., 0.27; and 315 min., 0.09. Therefore, signs of 1080 poisoning had appeared at a time when the blood ethanol concentration was still relatively high. We did not attempt further to treat 1080 poisoning in dogs with ethanol, since a marked effect of this substance on 1080 poisoning in dogs seemed unlikely.

The convulsant effect of 1080 in dogs influenced us to employ pentobarbital anesthesia in spite of the failure to demonstrate any beneficial effect on mice and guinea pigs. Chenoweth and St. John (10) have reported that pentothal or pentobarbital depress or obliterate the *petit mal* and *grand mal* types of electrical activity of the cerebral cortex seen in dogs after fluoroacetate administration. Ten dogs were poisoned with 0.1 mgm./kgm. of 1080. At the onset of the first convulsion each was given 35 mgm./kgm. of pentobarbital intravenously. Without exception the convulsions ceased, although in some instances the dogs continued for five to ten minutes to make running movements.[4] Pentobarbital was then administered as required to keep the dogs lightly anesthetized for eighteen to twenty-four hours. After eighteen hours no further pentobarbital was administered except when a dog showed a tendency to convulse on recovering from the anesthetic. Only $\frac{4}{10}$ of these dogs died. Comparison with previous experiments (1, 9) indicates a significant effect on mortality, but further experiments are required.

Discussion. Whether the measures reported here for treatment of 1080 poisoning in mice and guinea pigs are effective through increasing tissue metabolism, otherwise inhibited by 1080, it is impossible to say with certainty. This, however, is a most attractive working hypothesis, and no satisfactory alternative has presented itself to us. The known resistance of ethanol oxidations to inhibition by 1080 and the finding that ethanol catalyzes the oxidation of acetate by 1080 poisoned cells support the hypothesis. If the finding (5) that 1080 inhibits reactions leading to resynthesis of phosphocreatine is invoked, one can conceive this as a common factor in the effects of 1080 on nerve and on heart musculature, and the effects of increased metabolism to be exerted through promoting resynthesis of this substance.

It is somewhat surprising that a single dose of ethanol or ethanol plus acetate administered soon after 1080 poisoning suffices to effect the survival of the animals. Almost certainly the amounts of these substances given are soon exhausted. We wish to point out the analogy with experiments with yeast (7) in which an oxidative level, once attained, continues in spite of exhaustion of the ethanol which catalyzed its attainment. Possible explanations of this have been discussed previously (7).

Various non-specific effects of the treatment procedure such as anesthetic effects of ethanol, alkalizing effects of sodium acetate, and acidifying effects of acetic acid produced by oxidation of ethanol to acetic acid would not appear to be

[4] Cf. Chenoweth and Gilman (1), p. 98.

involved on the basis of total results. Small amounts of ethanol suffice when acetate is provided. Anesthesia, at least with pentobarbital, is ineffective except in dogs. The sodium of sodium acetate is unnecessary.

We should like to point out that ethanol is of varying usefulness in the species studied. It counteracts some two times the LD_{50} in mice, saves significant numbers of guinea pigs and rabbits from doses barely greater than the LD_{50}, but is probably of little benefit to dogs.

We have considered the possibility of a correlation existing between the mechanism of death in various animal species and the efficacy of ethanol. However, of the two species succumbing to central nervous effects (1) guinea pigs respond well to ethanol while dogs probably are not benefited. Rabbits, in which the action of fluoroacetate is on the heart, are also protected by ethanol to about the same extent as guinea pigs. The mechanism causing death in mice is not clear. Chenoweth and Gilman (1) did not study this species. While mice poisoned with the large doses used in most of our studies died following a convulsive seizure, we have not established the cause of these convulsions. Either direct effects of fluoroacetate on the brain or the anoxia resulting from a poisoned heart might be responsible. On the whole, we favor the idea that the effects of ethanol are not dependent on the organ (brain or heart) affected.

It is, however, interesting to consider the fact that mice, most resistant of the species studied to the action of fluoroacetate, respond best to ethanol administration, while dogs, which are most susceptible to the poison, do not. Guinea pigs and rabbits, intermediate in susceptibility to fluoroacetate, lie between the other two species as regards response to ethanol. The underlying basis of this apparent correlation is not clear, but one may speculate that resistance depends on the supply of some metabolite. In a species in which this metabolite is easily exhausted either because of rapid breakdown or slow synthesis, we might also expect to encounter difficulty in catalyzing the formation of a sufficient additional amount of the metabolite.

Because we do not plan to extend the observations reported here, and because previous informal reports of the results have served in at least one instance as a basis for treating a child poisoned with 1080[5], we wish to state clearly our opinions regarding the potential therapeutic efficacy of the measures reported. Ethanol administered before signs of 1080 poisoning appear is doubtless effective in three species, mice, guinea pigs, and rabbits. Its value to dogs is not established. Pentobarbital is of significant but minor value in dogs, saving only a small percentage of animals from about an LD_{75}. There is no evidence that ethanol and pentobarbital combined are better than either alone in a given species, and the combination may indeed be harmful. There is no evidence that ethanol alone, given soon after 1080 poisoning, will be harmful and it may be of value. We prefer to look upon the results as being of value chiefly as a contribution to understanding the mechanism of action of 1080.

[5] Personal communication, Dr. Eleanore R. Wright. Both ethanol and pentobarbital were used, the latter to control convulsions. The child survived.

SUMMARY

1. The LD_{50} of subcutaneously injected sodium fluoroacetate (1080) for mice is 19.3 mgm./kgm. with fiducial limits (p = 0.01) of 15.8 and 23.7 mgm./kgm.

2. The LD_{50} of 1080 injected intraperitoneally into guinea pigs is 0.378 mgm./kgm. with fiducial limits of 0.347 and 0.406 mgm./kgm. On a molar basis sodium fluoroacetate and methyl fluoroacetate are equally toxic for guinea pigs.

3. The LD_{50} of 1080 injected subcutaneously into rabbits is 0.281 mgm./kgm. with fiducial limits of 0.240 and 0.340 mgm./kgm.

4. Mortality among 1080 poisoned mice, guinea pigs, and rabbits can be significantly reduced if about 800 mgm./kgm. of ethanol are administered subcutaneously as a 10 per cent ethanol solution in normal saline within thirty minutes of poisoning. The most striking effects are obtained in mice when the ethanol is given within ten minutes of poisoning.

5. Light pentobarbital anesthesia maintained for eighteen to twenty-four hours significantly reduced mortality among dogs poisoned with 0.10 mgm./kgm. of 1080.

6. The following substances had no significant effect on mortality resulting from 1080 poisoning of mice: citrate, succinate, fumarate, malate, glycerol, pyruvate, glycine or glutamic acid in doses of 500 mgm./kgm. injected subcutaneously; or dinitrophenol in a dose of 5 mgm./kgm.

7. The mechanism of protection against fluoroacetate poisoning by ethanol is discussed.

REFERENCES

1. Chenoweth, M. B., and Gilman, A.: This Journal, **87**: 90, 1946.
2. Bartlett, G., and Barron, E. S. G.: J. Biol. Chem., **169**: 67, 1947.
3. Kalnitsky, G., and Barron, E. S. G.: J. Biol. Chem., **170**: 83, 1947.
4. Chenoweth, M. B.: Personal communication.
5. Colowick, S. P.: Personal communication.
6. Hutchens, J. O., and McMahon, T. M.: Fed. Proc., **6**: 264, 1947.
7. Black S., and Hutchens, J. O.: Arch. Biochem., **17**: 211, 1948.
8. Bliss, C. I.: Quart. J. and Yearbook of Pharm., **10**: 192, 1938.
9. Coon, J. M.: Unpublished data.
10. Chenoweth, M. B., and St. John, E.: This Journal, **89**: 76, 1947.

THE HISTAMINE ACTIVITY OF SOME β-AMINOETHYL HETEROCYCLIC NITROGEN COMPOUNDS

HENRY M. LEE AND REUBEN G. JONES

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Indiana

Received for publication September 29, 1948

The renewed interest in the physiological effects of histamine and its possible role in clinical allergies has stimulated a great amount of investigation of various histamine antagonists. In order to gain some insight into the fundamental mechanisms involved in the activity of histamine we have tested twenty-four compounds which are more or less closely related to histamine. Most of these substances are new, and their synthesis and characterization will be reported elsewhere. Literature references to those compounds which are not new are given in tables I and II. Each of the compounds has a β-aminoethyl side-chain attached to an aromatic, heterocyclic, nitrogen-containing nucleus. By a consideration of the physical and chemical properties of these various heterocyclic nuclei it was hoped that some correlations between chemical constitution and histamine activity might be drawn.

Previous workers (1–6) have examined the activity of a number of analogues obtained by attaching substituents to the side-chain and to the imidazole nucleus of histamine. More recently it has been observed that 2-β-aminoethylpyridine (7, 8) and 3-β-aminoethylthiazole (9, 10) also exhibit typical histamine activity. *We have now found that a variety of other β-aminoethyl heterocyclic compounds* exhibit typical histamine activity.

METHODS. The various physiological effects of histamine are too well known to be mentioned here. To estimate histamine-like activity we chose two tests which were simple and which illustrated two different functions of histamine. These were the spasmogenic effect on the isolated guinea pig ileum and the depression of blood pressure in the anesthetized cat. The isolated smooth muscle strips were arranged to record in the usual Magnus manner while suspended in Tyrode's solution. First the tissue was exposed to varying concentrations of histamine acid phosphate until a concentration was found which would give uniform submaximal responses on repetition. These varied from 2×10^{-3} to 10×10^{-3} microgm. per cc. Solutions of the histamine analogues were then tested until a concentration was found which would match the histamine response. By comparing doses of histamine and analogue which produced equal effects a ratio of activity was obtained. As a check on the specificity of histamine activity, the antagonistic action of an antihistamine agent, thenylpyramine hydrochloride (Histadyl Hydrochloride), to the analogues was observed. Preliminary two-minute exposure of the isolated ileum to the antagonist, 0.5–1.0×10^{-3} microgm. per cc. was usually sufficient to inhibit the effect of histamine acid phosphate to the extent of 75 per cent or greater in the above mentioned concentrations. The analogue was considered to be active on this tissue if it caused a contractile response which was inhibited by the antihistaminic agent to the same degree as histamine.

Similarly the responses to the intravenous injection of the analogues were observed and compared with the transient depressor effects induced by histamine acid phosphate in the anesthetized cat. The animal was given a sufficient dose of histamine acid phosphate to cause a suitable response. Then increasing doses of the analogue to be tested were injected

TABLE I

Compounds possessing histamine activity

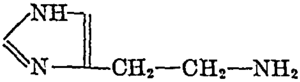
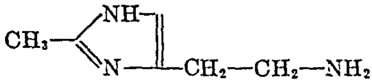
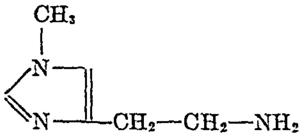
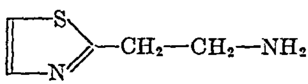
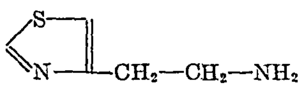
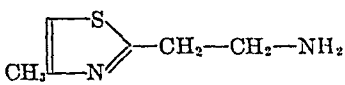
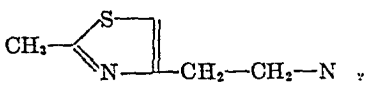
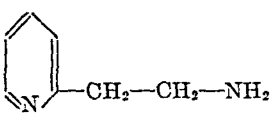
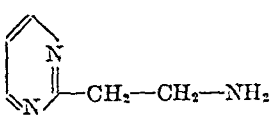
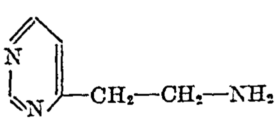
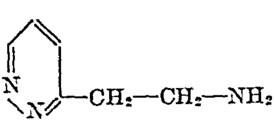
NO.	STRUCTURAL FORMULA		ACTIVITY	
			Guinea pig intestinal strip	Cat blood pressure
I			1.00	1.00
II		(2)	0.30	0.15
III		(13)	0.006	0.002
IV			0.3	0.08
V		(9)	0.02	0.006
VI		(19)	0.15	0.02
VII		(10)	0.005	0.003
VIII		(7)	0.09	0.02
IX			0.05	0.02
X			0.0009	0.002
XI			0.002	0.0015

TABLE II

Compounds possessing no histamine activity

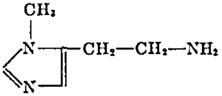
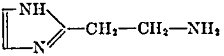
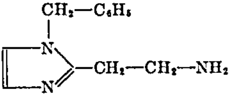
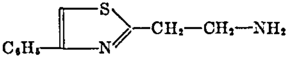
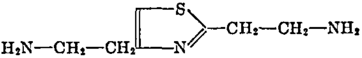
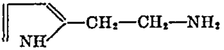
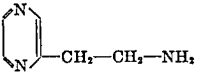
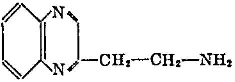
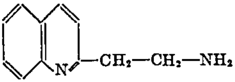
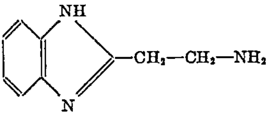
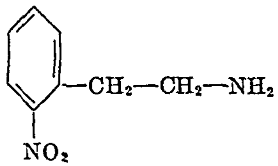
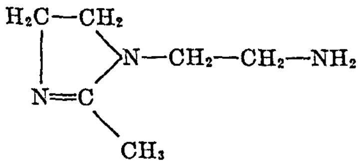
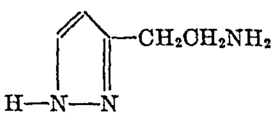
NO.	STRUCTURAL FORMULA	
XII		(13)
XIII		
XIV		
XV		
XVI		
XVII		
XVIII		
XIX		
XX		
XXI		(20)

TABLE II—*Concluded*

NO.	STRUCTURAL FORMULA
XXII	 (21)
XXIII	
XXIV	

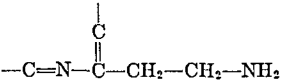
until a response equal to that of the histamine dose was obtained. The activity of the analogue was expressed as the ratio of the doses of histamine and analogue which produced equal effects. As previously reported (11) the preliminary intravenous injection of the histamine antagonist, 'Histadyl Hydrochloride,' 1.0 mgm. per kgm., will completely abolish the effect of subsequent injections of small doses of histamine acid phosphate, 0.5–1.0 mgm. This procedure likewise was used to check the specificity of histamine-like activity of the analogue. Each compound was evaluated by both tests, and each test was performed two or more times.

RESULTS. The compounds on which we are reporting at this time have been divided into two groups. Those which have histamine activity are presented in table I, and those which have no histamine activity are presented in table II. The relative potencies of the compounds of table I are expressed as the ratio: molar concentration of histamine/molar concentration of compound necessary to produce equal effects.

DISCUSSION. It was hoped that some correlation between histamine activity and a common physical property of the compounds might be observed. One physical property which was measured was the basicity of the two basic centers present in each of the substances. These basicities, expressed as pKα values, fell in the range 9.1 to 9.9 for the primary amino group on the ethyl side-chain. Incidentally the pKα value of another type of physiologically active compound, β-phenylethyl amine, is 9.83 (12). The pKα values of the aromatic portions of the molecules varied over the range 1.4 to 6.5. It is not surprising that no obvious correlations between histamine activity and pKα values appeared. The basicities and other physical properties of this series of compounds are being studied further.

Based upon their studies of the β-aminoethyl derivatives of pyridine, Walter,

Hunt and Fosbinder (7) were led to suggest that the minimum requirements for histamine activity were embodied in the structural fragment [A]. Niemann



[A]

and Hays (8) further concluded that this fragment should be represented as [B], in which the bond distances, a, b, c, have the values 1.36 ± 0.01Å, 1.38 ± 0.02Å and 1.40 ± 0.01Å, respectively. In view of the results presented in tables

$$\text{—CH—a—N—b—C(CH}_2\text{CH}_2\text{NH}_2\text{)—c—CH—}$$

[B]

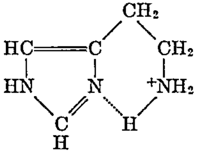
I and II it is obvious that the fragment [A] or [B] does not represent the minimum requirements for histamine activity. The one structural feature which is common to all of the active compounds that we have tested so far is the system [C] in which the portion —N=C— or =N—C— is part of an aromatic nucleus.

$$\text{—N=C—CH}_2\text{CH}_2\text{NH}_2 \quad \text{or} \quad \text{=N—C—CH}_2\text{CH}_2\text{NH}_2$$

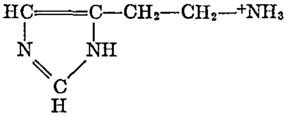
[C]

It will be seen that system [C] is a contraction of [A]. The presence of the structural fragment [C] does not guarantee that a compound will have histamine activity. Most of the inactive compounds (table II) also have the system [C] in their structures.

Niemann and Hays have asserted that of the two tautomeric forms of histamine [D] and [E] only [D] is active, and this is because only [D] is capable of forming an intramolecular hydrogen bond between the side-chain and nuclear



[D]



[E]

nitrogen atoms. So far as we are aware there is no theoretical basis for assuming that intramolecular hydrogen bonding has anything to do with histamine activity. However, it is interesting to note that all of the active compounds (table I) which we have tested are structurally favorable for hydrogen bond formation. On the other hand, many of the compounds which did not have any histamine activity, notably, numbers XIII, XVIII, XX, and XXIV, also are

capable of the same type of intramolecular hydrogen bonding. The two methylated derivatives of histamine, compounds III and XII, are of interest in this connection. Both of these substances (XII in the form of its picrate salt) were synthesized by Pyman (13) who stated that neither had any significant activity. We have now found that although compound XII has no activity, compound III definitely does have histamine activity. These results appear to support the contention of Niemann and Hays that hydrogen bonding (which is possible in III but not in XII) is an important factor in determining whether or not a compound has histamine activity. However, compound III contains the fragment [C] in its structure whereas the inactive compound XII does not.

The size and shape of the aromatic nucleus appears to have a definite bearing upon the activity of the various compounds. Substitution of a methyl group into the 2-position of histamine (compound II) lowered the activity several fold, but substitution of the methyl group into the 1-position (compound III) lowered the activity several hundred fold. A methyl group in the thiazole nucleus (compounds VI and VII) decreased the activity, and a phenyl group in the same thiazole nucleus (compound XV) completely abolished activity. The inactive compound XX may be regarded as a nuclear-substituted derivative of the active compound VIII.

The spatial disposition of the various atoms in the aromatic ring and the nature of the atoms themselves have a profound influence upon the histamine activity. Compound XIII, which is an imidazole derivative isomeric with histamine, is completely inactive. On the other hand compound IV is one of the most active found, and it may be looked upon as an isoster of the inactive compound XIII in which the —NH— portion of the ring has been replaced with —S—. The β-aminoethyldiazines (compounds IX, X, XI and XVIII) comprise an interesting group. Compound XVIII, which is the only inactive one of this series, differs from the others only in the position of one of the nitrogen atoms in the ring. In trying to visualize the nature of the various aromatic rings it is difficult to apply any considerations of bond distances because these values are reliably known for only three of the nuclei, namely those in compounds VIII, XVII and XVIII (14). The bond distances and bond angles of the pyridine ring in compound VIII are almost identical with those of the pyrazine ring in compound XVIII, yet one of the compounds is highly active while the other is completely inactive.

Two of the compounds, XVII and XXII, had an appreciable pressor activity. This is not entirely unexpected because compound XXII is a substituted phenylethylamine, and compound XVII, 2-β-aminoethylpyrrole, may be regarded as an isoster of β-phenylethylamine. Compound XXII was tested for histamine activity with the idea that it might closely resemble compound VIII, since nitrobenzene recently has been described as isosteric with pyridine (15). 2-β-Aminoethylpyrrole has in its structure an arrangement of atoms corresponding with fragment [C] but it does not have, in the aromatic portion of the molecule, an arrangement of bonds corresponding with [C].

No correlations can be drawn between histamine activity and the intrinsic

chemical properties of the aromatic nuclei in the present series of compounds Two general types of nuclei are represented The six membered ring systems including pyridine, pyrazine, pyrimidine and pyridazine are relatively weakly nucleophylic They undergo substitution reactions such as halogenation and nitration with great difficulty, and they are highly resistant to oxidation but quite susceptible to reduction The five membered ring systems, imidazole and pyra zole, are relatively strongly nucleophylic in that they are easily halogenated and nitrated but are highly resistant to reduction Both types of nuclei are to be found among the active compounds, and both types are also to be found among the inactive compounds

CONCLUSIONS There is no doubt that a combination of many different factors is responsible for the physiological activity of drugs On the basis of the present data it appears that one important requirement for histamine activity is the presence of the fragment $-N=\overset{|}{C}-CH_2-CH_2-NH_2$ or $=N-\overset{\|}{C}-CH_2-CH_2-NH_2$ in the molecule A fair test of this requirement has probably not been made, however, because only a few compounds have been examined which did not contain this structural feature It appears that the size and shape of the aromatic nucleus is a determining factor in histamine activity The highly active compounds are those with small and unsubstituted nuclei Closely associated with the size and shape is the arrangement of the atoms within the aromatic portion of the molecule These architectural features of the molecules are probably responsible for the ability of the drugs to be adsorbed on the receptor centers of the cell surfaces (16) The mechanism of this adsorption process is most obscure Even the use of such refined physical chemical measurements as bond distances and bond angles gives us no clearer picture as to why some com pounds have histamine activity while others do not In certain other series of physiologically active substances a consideration of bond distances has been used to great advantage in correlating activity with chemical structure (17, 18)

SUMMARY

A series of twenty four β aminoethyl heterocyclic nitrogen compounds has been tested for histamine activity Eleven of these compounds are active Some possible relationships between histamine activity and chemical constitution have been discussed

REFERENCES

1 DALE, H H, AND DUDLEY H W THIS JOURNAL, 18 103, 1921
2 TAMAMUSHI, Y J Pharm Soc Jap, 53 226 1933
3 VARTIAINEN, A THIS JOURNAL 54 265 1935
4 SCHNEDORF J G, AND IVY, A C Proc Soc Exp Biol and Med, 32 777, 1934–1935
5 ACKERMANN, D, AND WASMUTH, W Hoppe Seyler's Ztschr f physiol Chem, 259 28, 1939
6 ALLES G A, WISEGARVER B B, AND SHULL, M A THIS JOURNAL, 77 54 1943
7 WALTER, L A HUNT W H, AND FOSBINDER, R J J Am Chem Soc, 63 2771, 1941
8 NIEMANN C AND HAYS J T J Am Chem Soc, 64 2288, 1942
9 ERLENMEYER, H, AND MULLER M Helvet Chim Acta, 28 922, 1945

10. Burger, A., and Ullyot, G. E.: J. Org. Chem., **12**: 342, 1947.
11. Lee, H. M., Dinwiddie, W. G., and Chen, K. K.: This Journal, **89**: 83, 1947.
12. Carothers, W. H., Bickford, C. F., and Hurwitz, G. J.: J. Am. Chem. Soc., **49**: 2908, 1927.
13. Pyman, F. L.: J. Chem. Soc. London, **99**: 2172, 1911.
14. Schomaker, V., and Pauling, L.: J. Am. Chem. Soc., **61**: 1769, 1939.
15. Erlenmeyer, H., Aeberli, M., and Sorkin, E.: Helv. Chim. Acta, **30**: 2066, 1947.
16. Clarke, A. J.: Hefter's Handbuch der Experimentellen Pharmakologie; Springer, Berlin, 1937; vol. 4.
17. Schueler, F. W.: Science, **103**: 221, 1946.
18. Pfeiffer, C. C.: Science, **107**: 94, 1948.
19. Goldberg, A. A., and Kelly, W.: J. Chem. Soc. London, 1372, 1947.
20. Chatterjee, B.: J. Chem. Soc. London, 2965, 1929.
21. Johnson, T. B., and Guest, H. H.: Am. Chem. J., **43**: 310, 1910.

STUDIES ON THE TOXICITY AND MECHANISM OF ACTION OF p-NITROPHENYL DIETHYL THIONOPHOSPHATE (PARATHION)

KENNETH P. DuBOIS, JOHN DOULL, PAUL R. SALERNO, AND JULIUS M. COON

The University of Chicago Toxicity Laboratory[1] *and the Department of Pharmacology,*[2] *University of Chicago, Chicago, Illinois*

Received for publication September 30, 1948

The insecticidal action of p-nitrophenyl diethyl thionophosphate (parathion; E 605; Thiophos 3422) which was first observed in Germany has recently attracted considerable attention in this country because of the effectiveness of the compound for the extermination of plant insects. The possible widespread use of parathion as an insecticide stimulated our interest in obtaining data on its toxicity and pharmacologic action in mammals since no such information concerning this compound was available.

Recent studies on other phosphate esters (1–5) with insecticidal action such as hexaethyl tetraphosphate (HETP) and tetraethyl pyrophosphate (TEPP) have shown that these substances exert a cholinergic action in mammals. Preliminary studies in this laboratory (6) have indicated that parathion also exerts a cholinergic action as evidenced by symptoms typical of parasympathetic stimulation, cholinesterase inhibition, and a protective action by atropine against some of its actions. Other preliminary investigations (7, 8) have also suggested a similarity in the action of parathion and parasympathomimetic drugs.

Information concerning the toxicity and mechanism of action of parathion in mammals was considered essential in ascertaining the possible dangers associated with the use of this material as an insecticide. The present communication contains the results of more extensive studies on the toxicity of parathion and its pharmacologic action. Toxicity measurements were carried out on several species and the inhibitory action of parathion on cholinesterase was studied in rats under various conditions of treatment with parathion. The action of parathion on the blood pressure of cats and dogs was measured and observations were made on the responses of the bronchioles and the isolated and *in situ* small intestine. The present studies, which have demonstrated that parathion exhibits a high toxicity and strong cholinergic action in all of the species upon which it was tested, suggest that precautions should be taken to avoid undue exposure of men and domestic animals during the use of this agent as an insecticide.

METHODS. Adult male and female Sprague-Dawley rats (180–220 grams) were employed except where the effect of age on susceptibility to parathion was measured, in which case

[1] The work described in this paper was done in part under contract between the Medical Division, Chemical Corps, U. S. Army and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

[2] Partial support for this work was also derived from a contract with the Office of Naval Research, N6 ori-20, Task Order 11.

Symptoms. After intraperitoneal injections of parathion in mice and rats symptoms appeared in 2 to 5 minutes and death occurred in 5 to 24 minutes. Survivors of the LD_{50} were free from symptoms in 2 to 3 hours except for an apparent depression from which recovery was complete the following day. There were no delayed deaths in mice, though an occasional one occurred in rats as long as 3 days after poisoning. Symptoms in these animals started with an increase in respiration, followed soon by unsteadiness, lack of coordination, and scattered muscular twitches. Defecation, urination, lacrimation, and salivation occurred regularly. The severity of symptoms rapidly increased to prostration with generalized muscular fibrillations, body twitchings and tonic and clonic convulsions, followed by death apparently due to respiratory failure of peripheral origin. The heart continued to beat 2 to 3 minutes after cessation of respiration. There were numerous survivors, especially among the mice, of individuals whose symptoms had advanced to the convulsive stage.

The course of acute symptoms in cats and dogs following the intraperitoneal injection of parathion was essentially similar to that seen in rats and mice in severely poisoned cases. However, cats and dogs frequently displayed only mild acute symptoms and died more than 24 hours after poisoning. Animals succumbing in this manner exhibited first an apparent central depression with a deepened respiration, slight unsteadiness, scattered muscular twitchings and mild tremors. Food and water were left untouched. The animals appeared disoriented. A few individuals displayed intermittent mild convulsive seizures. The depression developed gradually to extreme weakness and prostration, with continued mild tremors and muscular twitchings. The respiration and heart action slowly became weaker until death intervened.

Cats and dogs dying in three hours or less showed severe symptoms of central and parasympathetic stimulation, which appeared 15 to 20 minutes after injection. First there appeared a restlessness with a beginning incoordination. The rate and depth of respiration were increased. Vomiting, defecation, urination, lacrimation, and copious salivation occurred regularly. Pupillary changes were inconstant, dilatation appearing in some animals, and a slight miosis or no observable change in others. Disorientation, tremors, muscular twitching and fibrillations, and prostration with clonic convulsions led to death with the cardiac action outlasting the respiration by 2 to 3 minutes. Before cessation of respiration stethoscopic examination revealed no alterations in heart action except an increase in rate accompanying the increase in muscular activity. Numerous moist rales could be heard throughout the chest.

Gross pathologic examination of cats and dogs immediately after the acute deaths usually showed an intensely contracted intestinal tract and urinary bladder. The lungs were congested and hyperemic and a small amount of foamy fluid was typically found in the trachea and bronchi. No other abnormalities were found. In the cats dying 2 to 3 days after the administration of parathion bronchopneumonic processes were observed, probably a result of aspirated saliva and vomitus. This was not considered an important factor in the fate of the animal since cats treated with atropine were protected against these lung changes but not against delayed death following equivalent doses of parathion.

Subacute toxicity of parathion to rats. The possibility of repeated exposure to sublethal doses of parathion during its use as an insecticide made it desirable to ascertain whether the compound exerted a cumulative action. Female rats were therefore given daily intraperitoneal injections of parathion for 20 days and the mortality was observed. After single doses deaths rarely occurred later than four hours following the administration of the compound. A cumulative action of the insecticide would, therefore, be indicated by death of the animals after successive sublethal doses of the insecticide given at 24-hour intervals. The mortality data obtained by giving repeated daily doses of parathion to rats are shown in table 2.

The results of this experiment demonstrated that daily sublethal doses of parathion exert a cumulative toxic action in rats. After daily intraperitoneal doses of 3 mgm./kgm. of parathion, which is below the LD_{50} dose (4 mgm./kgm.), none of the animals survived more than five doses of the drug. Daily doses of 1 and 2 mgm./kgm. of parathion for 10 days resulted in 46 per cent and 87 per cent mortality, respectively. The symptoms preceding death of the animals were similar to those observed in acutely poisoned rats. Food consumption by these animals showed no significant decrease as compared with normal rats. However, there was a definite inhibition of the growth of animals treated with parathion. After daily administration of 1 mgm./kgm. of parathion the average gain in weight was 4 per cent as compared with 10 per cent for the control animals. Daily doses of 2 mgm./kgm. of parathion resulted in an average loss of 11 per cent of the weight of 200-gram rats during the 10-day observation period. The results of these experiments indicated that continued exposure to sublethal doses of parathion results in subacute poisoning in rats and suggests the possibility of a cumulative action by parathion in other animals after continued exposure to the insecticide.

TABLE 2

The cumulative action of parathion in rats

DAILY DOSE OF PARATHION	DAYS AFTER FIRST INJECTION											MORTALITY 10 DAYS	MORTALITY 10 DAYS
	1	2	3	4	5	6	7	8	9	10	10–20		
	Mortality												
mgm./kgm.													%
3	6	3	3	2	1							15/15	100
2	1	4	3	3	1	1	0	0	0	0	1	13/15	87
1	0	0	2	3	0	0	2	0	2	2	2	11/24	46
0.5	0	0	0	0	0	0	0	0	0	0	0	0/5	0

Pharmacodynamic observations. In the cat and dog anesthetized with sodium phenobarbital (150 to 180 mgm./kgm. intraperitoneally) blood pressure responses to intravenous parathion, measured with a mercury manometer connected to a cannulated carotid artery, were irregular. A dose of 2 to 4 mgm./kgm. in either animal produced either a slight rise of 10 to 15 mm. Hg pressure requiring 10 to 20 minutes to reach a maximum, or a sudden fall of 10 to 20 mm. Hg followed by a

gradual slight rise above normal. On some occasions a pure fall of blood pressure was noted. These doses of parathion diminished by 10 to 25 times the amount of acetylcholine required to produce a blood pressure fall of 20 to 30 mm. Hg. A second injection of 2 to 4 mgm./kgm. of parathion was followed in 10 to 15 minutes by a cessation of respiration accompanied by a fall of blood pressure which was sometimes preceded by an evanescent anoxic rise. Artificial respiration instituted at this point prevented the death of the animal, but a moderate depressor effect was still observed. After further injections of parathion a marked bradycardia supervened, the tracing showing a greatly exaggerated pulse pressure suggesting an A-V block. These effects were readily reversed or prevented by atropine or procaine. In two atropinized dogs under artificial respiration almost indefinite amounts of parathion, up to 140 mgm./kgm. in divided doses, were given without visible effects on the animal except for scattered muscular twitchings. Atropine alone, however, protected against only 2 to 3 lethal doses.

Tracings taken from a pleural cannula inserted into the chest in 1 cat and 3 dogs under artificial respiration indicated that parathion, in doses of 3 to 5 mgm./kgm., induced a moderate bronchial constriction.

The isolated intestinal muscle (ileum) of the rabbit was highly sensitive to parathion and to its oxygen analog. In minimal molar concentrations of 27.5×10^{-8} and 5.4×10^{-8}, respectively, these drugs induced in the intestinal strip a typical abrupt change from the normal pendular rhythm to a slow "peristaltic" type of rhythm of greater amplitude. Thus in this respect the relative potencies of the two drugs were roughly parallel to their relative toxicities.

Records made of the movements of the small intestine of 1 cat and 2 dogs *in situ*, using the device described by Jackson (13) showed a persistent increase in tone and activity in response to the intravenous administration of 2 to 4 mgm./kgm. of parathion.

The isolated perfused heart of the rabbit showed little specific sensitivity to parathion or its oxygen analog. When these agents were perfused through the heart in a concentration of 1:50,000 in Ringer-Locke solution there was no immediate effect, but a gradual diminution in amplitude without marked change in rate, until the excursions became imperceptible about 5 minutes after the beginning of the perfusion of the drugs. Concentrations of 1:200,000 in the perfusing fluid were tolerated without effect for one hour. Both drugs lacked any tendency to augment the response of the isolated heart to acetylcholine. The action of these drugs on the isolated heart thus appeared to be a direct toxic one, wholly independent of their anticholinesterase properties.

Inhibitory action of parathion on cholinesterase. In view of the cholinergic action of parathion in mammals it was of interest to investigate its effect on cholinesterase. In order to ascertain the effect of parathion on cholinesterase *in vitro* several concentrations of the compound were tested on rat brain cholinesterase. The inhibitor was dissolved in the Ringer-bicarbonate buffer and incubated in the Warburg vessels with 50 mgm. of homogenized whole rat brain throughout the gassing and equilibration period before addition of the acetyl-

choline. Under these test conditions a final concentration of 1.2×10^{-6} *M* parathion produced 50 per cent inhibition of rat brain cholinesterase *in vitro*. In contrast to the strong inhibitory action of the compound on cholinesterase it had no inhibitory effect *in vitro* on the oxidation of glucose nor on anaerobic glycolysis in brain homogenates. No observable changes in the concentrations of the acid-soluble intermediates of glycolysis could be detected in the brain tissue from rats at the time of death after the intraperitoneal administration of 20 mgm./kgm. of parathion.

Substitution of the sulfur of parathion by oxygen greatly enhanced the inhibitory action on cholinesterase. The oxygen analog of parathion, diethyl p-nitro phenylphosphate, produced 50 per cent inhibition of cholinesterase at a final concentration of 5×10^{-9} *M*. The greater inhibitory action of this compound on cholinesterase is consistent with its greater toxicity to mammals as compared with parathion.

To test the effect of parathion on cholinesterase *in vivo* 5 rats were given 20 mgm./kgm. of parathion and the brain and submaxillary glands were removed for cholinesterase measurements at the time of death in about eight minutes after poisoning. After this dose of parathion the average cholinesterase activity of brain tissue fell to 8 per cent of normal while the enzyme activity of the submaxillary glands decreased to 22 per cent of normal. After 5 mgm./kgm. of parathion the average cholinesterase activity of brain was 15 per cent of normal while the submaxillary glands exhibited 29 per cent of the control activity at the time of death. Thus, parathion strongly inhibited both the central and peripheral cholinesterase of rats.

From the results of cholinesterase measurements it appeared likely that lethal doses of the insecticide would cause a considerable elevation of the free acetylcholine of rat brain. To ascertain the extent of the rise in free acetylcholine of brain after parathion 4 rats were given 20 mgm./kgm. of parathion intraperitoneally and the brains were quickly removed at the time of death for the measurement of free acetylcholine. Four normal rats were sacrificed by decapitation and the acetylcholine values were obtained for comparison with the poisoned animals. Whereas the free acetylcholine of the brain of normal rats ranged from 0.79 to 0.96 micrograms/gram (average 0.86 micrograms/gram) the values for poisoned animals were 3 to 3.8 micrograms/gram (average 3.4 micrograms/gram). These findings demonstrated that a marked rise in free acetylcholine of the brain tissue of rats accompanied the depression of cholinesterase produced by parathion.

Reversibility of the inhibition of cholinesterase by parathion. In order to determine whether the inhibitory action of parathion on cholinesterase is reversible male rats were given 5 mgm./kgm. of parathion and groups containing 3 animals each were sacrificed at various intervals from 0.5 to 4 hours after administration of the drug. Cholinesterase measurements were performed on the homogenized whole brain of each animal.

It was of interest to compare the duration of the inhibitory action of parathion on cholinesterase with that of other phosphate esters. It has been well es-

tablished previously by many investigators that di-isopropyl fluorophosphate (DFP) produces an almost completely irreversible inhibition of cholinesterase. However, the duration of the inhibitory action of TEPP has received little attention previously. While the rapid recovery of animals receiving sublethal doses of TEPP suggests a reversible inhibition by this agent it was recently reported (14) that plasma esterase is irreversibly inactivated *in vitro* by TEPP. We therefore compared the duration of action of parathion and TEPP on brain cholinesterase of rats. A group of animals was given 0.5 mgm./kgm. of TEPP and sacrificed at various times after the administration of the compound for brain cholinesterase measurements. The reversibility of the inhibitory action

TABLE 3

Reversibility of the inhibition of rat brain cholinesterase by parathion and TEPP

HOURS AFTER PARATHION OR TEPP	CHOLINESTERASE ACTIVITY	CONTROL ACTIVITY
	Parathion (5 mgm./kgm.)	
	cmm. CO_2*/50 mgm. brain/10 min.*	*per cent*
Control	103	
0.5	6	5.8
1	11	10.7
2	58	57.5
4	100	97.0
	TEPP (0.5 mgm./kgm.)	
0.5	24	23.3
1	42	40.7
2	49	47.5
4	71	69.2
6	88	85.5
8	95	92.3
16	99	96.0

of parathion and TEPP on cholinesterase is shown by the data in table 3 in which each value is the average of measurements on at least 5 animals.

From these data it may be seen that the inhibitory action of both parathion and TEPP on brain cholinesterase is reversible. Whereas the average cholinesterase activity was 6 per cent of normal in 0.5 hour after 5 mgm./kgm. of parathion it returned to 57.5 per cent of normal in 2 hours and to 97 per cent of normal in 4 hours. After 0.5 mgm./kgm. of TEPP the cholinesterase activity fell to 23.3 per cent of normal in 0.5 hour but 96 per cent of the normal activity was regained in 16 hours.

Effect of repeated daily doses of parathion on the brain cholinesterase of rats. The reversibility of the inhibitory action of parathion on cholinesterase activity after single sublethal doses raised the question as to the nature and extent of the cholinesterase changes in the brain tissue of rats which succumbed to repeated daily sublethal doses of the insecticide. Since single doses (5 mgm./kgm.) of

parathion caused a depression of cholinesterase which lasted only 4 hours it did not appear likely that its cumulative action was due to irreversible inactivation of the enzyme.

To measure the effect of repeated doses of parathion on cholinesterase activity rats were given 1 mgm./kgm. of parathion and groups of 3 animals were sacrificed 24 hours after each injection for brain and submaxillary cholinesterase measurements. Another series of animals were given the same daily dose of parathion and groups containing 3 animals each were sacrificed on different days 30 minutes after the last injection of parathion since the symptoms were at a maximum at this time after each injection. The results of these measurements are shown by the data in table 4.

TABLE 4

Effect of daily intraperitoneal administration of 1 mgm./kgm. of parathion on the cholinesterase activity of rat tissues

PARATHION TREATMENT	CHOLINESTERASE ACTIVITY	
	Brain	Submaxillary
24 hours after last injection		
days	*% of normal activity*	*% of normal activity*
1	98	100
2	94	100
3	77	92
5	50	70
6	24	43
0.5 hours after last injection		
1	45	
2	18	
3	12	
4	7	
5	6	

It may be seen from the data in table 4 that the cholinesterase activity of brain and submaxillary tissue returned to nearly normal values in 24 hours after the first two doses of the drug. However, further daily doses resulted in a progressive decrease in the activity of both the central and peripheral cholinesterase. When groups of animals were sacrificed at 30 minutes after each daily injection of parathion a progressive decrease in brain cholinesterase activity was also observed. Whereas the first injection caused a decrease in cholinesterase activity of brain to 45 per cent of normal the fourth daily dose of the drug caused a depression of the enzyme activity to 7 per cent of normal.

Measurements of the free acetylcholine content of the brain of rats sacrificed 30 minutes after daily doses of parathion showed a rise in free acetylcholine to 120 per cent of normal after the first dose of parathion, while after the fifth daily dose the free acetylcholine increased to 166 per cent of the normal value. This

greater rise in free acetylcholine after repeated daily doses of parathion is consistent with the progressive fall in cholinesterase activity after continued daily administration of the drug.

The results of this experiment indicated that the inhibition of cholinesterase produced by a single dose of parathion is reversible but continued daily administration of the drug results in a progressive loss of the catalytic activity of the enzyme. A progressive decrease in the cholinesterase activity of rat tissues to low levels is, therefore, associated with the cumulative toxic action of this insecticide in rats.

Effect of atropine and eserine on the inhibition of cholinesterase by parathion. The protective action (15) of eserine against poisoning by DFP has been explained (16) on the basis of competition by eserine for cholinesterase. According to this hypothesis eserine protects against irreversible inhibition by DFP by forming a reversible complex with the enzyme. If eserine protected against the lethal action of parathion by competitive inhibition it seemed possible that this might be demonstrable by cholinesterase measurements on the tissues of animals treated with eserine, parathion and a combination of the two drugs. Previous studies (17) have shown that eserine does not have a marked inhibitory action on brain cholinesterase *in vivo* but has a strong inhibitory action on peripheral cholinesterase.

To observe whether eserine exerted a protective action against inhibition of central and peripheral cholinesterase by parathion groups of rats were given either eserine, parathion, or eserine and parathion. Groups containing at least 6 rats were sacrificed one-half hour after the administration of parathion for cholinesterase measurements on brain and submaxillary tissue. The animals which received eserine without parathion were sacrificed after the same time interval following eserine as those which had also received parathion. Some of the animals were given atropine in addition to the other drugs since atropine was necessary for protection against the lethal action of parathion although it was not anticipated that atropine would exert an effect on cholinesterase. The animals were given 100 mgm./kgm. of atropine sulfate followed in 15 minutes by 5 mgm./kgm. of eserine salicylate. Thirty minutes after the eserine was administered the animals were given 5 mgm./kgm. of parathion and were sacrificed 30 minutes thereafter for the enzyme measurements. All of the drugs were administered intraperitoneally. The results of this experiment are shown by the data in table 5.

The results of this experiment demonstated that eserine exerted a marked protective effect against the inhibitory action of parathion. Parathion alone caused a marked depression of both brain and submaxillary cholinesterase while eserine alone produced only a 12 per cent decrease in brain cholinesterase activity and 49 per cent inhibition of submaxillary cholinesterase. The combination of parathion and eserine resulted in 37 per cent inhibition of brain cholinesterase and 45 per cent inhibition of submaxillary esterase as compared with 85 per cent and 49 per cent inhibition respectively of the two tissues from animals receiving only parathion. As was anticipated atropine had no influence on the cholines-

terase activity of the tissues. Thus, the protective action of eserine against acute parathion poisoning in rats is associated with a marked reduction in the amount of inhibition of brain cholinesterase by parathion, and a smaller protection against inhibition of peripheral esterase by the insecticide.

DISCUSSION. An investigation of the pharmacologic actions of parathion has demonstrated that this new insecticide exerts a cholinergic action in mammals. The symptoms which followed acute poisoning were typical of those produced by parasympathomimetic drugs. Pharmacodynamic observations gave further indications that parathion exerts a parasympathomimetic action. The augmentation by parathion of the depressor response to acetylcholine and the protective action of atropine against many of the effects of the drug point to a

TABLE 5

The effect of atropine and eserine on the inhibition of cholinesterase by parathion

GROUP	TREATMENT	DOSE OF DRUG	INHIBITION OF CHOLINESTERASE ACTIVITY	
			Brain	Submaxillary
		mgm./kgm.	%	%
1	Parathion	5	85	71
2	Eserine salicylate	5	12	49
3	Atropine sulfate Eserine salicylate	100 5	12	50
4	Eserine salicylate Parathion	5 5	37	45
5	Atropine sulfate Eserine salicylate Parathion	100 5 5	38	42

parasympathetic stimulation by this drug. Parathion appeared to lack the definite pressor action exhibited by other potent anticholinesterase agents such as eserine, neostigmine, HETP, and TEPP (3). However, this action is not considered to be necessarily associated with cholinesterase inhibiting properties in a drug. DFP also fails to show a consistent pressor action.

Testing the insecticide under a variety of conditions has demonstrated that it exerts a strong inhibitory action on cholinesterase. In contrast to the marked inhibitory action on this enzyme parathion exerted no inhibitory action on the oxidation of glucose nor on glycolysis by brain homogenates at concentrations much higher than those required to produce inhibition of cholinesterase. Both central and peripheral cholinesterase was inhibited *in vivo* by parathion indicating that this agent, like TEPP (5), readily gains access to the cholinesterase of the brain.

Unlike DFP the inhibitory action of parathion on cholinesterase is almost en-

tirely reversible. However, daily sublethal doses seemed to cause permanent inactivation of part of the enzyme eventually resulting in low cholinesterase activity at the time of death. The reversibility of the inhibition of cholinesterase by parathion is consistent with the rapid disappearance of symptoms when sublethal doses of the drug are given. Since a similar reversible action was exhibited by TEPP *in vivo* doubt is thrown upon the significance of the irreversible inactivation of plasma cholinesterase by this compound *in vitro* (14).

The data obtained in the present studies indicate that pre-treatment of rats with eserine markedly reduces the inhibition of brain cholinesterase by parathion and to a lesser extent reduces the inhibition of the peripheral esterase. It was postulated by Koelle (16) that eserine protected against DFP by combining with cholinesterase thereby preventing irreversible inhibition by DFP. It is likely that the mechanisms of the protective action of eserine against DFP and against parathion are the same.

Toxicity measurements showed that parathion exhibits a rather high toxicity to mammals with no marked species difference in susceptibility being noted. These findings indicate that care should be exercised to avoid acute poisoning in man. From the practical standpoint the cumulative toxic action of parathion is also of importance. Parathion is quite stable toward hydrolysis, thus care should be taken to avoid continued exposure to the toxic agent through ingestion of contaminated food and handling the material.

A sex difference in susceptibility as large as that observed in rats with parathion is an uncommon finding with cholinergic drugs. In the case of parathion it appears that the sulfur of the molecule is in some manner responsible for the sex difference since the oxygen analog of parathion, diethyl p-nitro phenylphosphate, was of equal toxicity to male and female rats. It was interesting that the sex hormones appeared to equalize the susceptibility of male and female rats to the insecticide. It has been shown (18) that there exists a significant difference in the serum cholinesterase activity of male and female rats with the latter sex exhibiting the higher activity. Several studies (19, 20, 21) have also demonstrated that the amount of non-specific cholinesterase activity in rat serum is influenced by sex hormones. Estrogenic hormones elevate the concentration of the enzyme while testosterone depresses it. On the basis of these findings it might be expected that diethyl stilbesterol would decrease the susceptibility of rats to parathion rather than exerting the opposite effect as was the case in our experiments. Further studies are, therefore, necessary to elucidate the exact manner in which the sex hormones influence the acute toxicity of parathion.

SUMMARY

1. Measurements of the acute toxicity of parathion given intraperitoneally gave the following approximate LD_{50} values in mgm./kgm.: male rats 7, female rats 4, mice 5–10, cats 3–5, and dogs 12–20. The LD_{50} by oral administration was 6 mgm./kgm. for female rats and 15 mgm./kgm. for male rats.

2. The LD_{50} for the oxygen analog of parathion, diethyl p-nitro phenylphosphate, was 1.2 mgm./kgm. when given intraperitoneally in rats and 3.5 mgm./kgm. when given orally with both sexes showing equal susceptibility.

3 Diethyl stilbesterol increased the susceptibility of male rats to parathion while testosterone propionate decreased the susceptibility of females to the insecticide

4 A cumulative toxic action by parathion was observed in rats after daily administration of sublethal doses of the drug

5 The symptoms produced by parathion were similar in all of the species tested and were typical of parasympathomimetic drugs Pharmacodynamic studies showed irregular blood pressure responses to intravenous injection, a slight fall or rise, or a diphasic response being seen on different occasions The depressor response to acetylcholine was augmented and atropine antagonized many of the effects of the drugs *After lethal doses of the drug death resulted* from respiratory paralysis

6 Parathion was found to be a strong inhibitor of cholinesterase *In vitro* a final concentration of 1 2 $\times 10^{-8}$ M parathion produced 50 per cent inhibition of rat brain cholinesterase After 5 mgm /kgm of the insecticide given intraperitoneally the cholinesterase activity of the brain was 15 per cent of normal while the submaxillary glands exhibited 29 per cent of control activity at the time of death The free acetylcholine rose markedly in the brains of poisoned animals

7 Treatment of rats with eserine before parathion resulted in partial protection of both the central and peripheral esterase against inhibition by the insecticide and a combination of atropine and eserine protected against the lethal action of the drug

REFERENCES

1 DuBois, K P, and Mangun, G H Proc Soc Exper Biol and Med, 64 137, 1947
2 Burgen, A S V, Keele, C A, Chennells, M, del Castillo, J, Floyd, W F, Slome, D, and Wright, S Nature, 160 760, 1947
3 Coon, J M, and Salerno, P R Fed Proc, 7 212, 1948
4 Dayrit, C, Manry, C H, and Seevers, M H This Journal 92 173, 1948
5 Mangun, G H, and DuBois, K P Fed Proc, 6 353, 1947
6 DuBois, K P, Doull, J, and Coon, J M Fed Proc, 7 216, 1948
7 Hagan, E C, and Woodard, G Fed Proc, 7 224, 1948
8 Hazelton, L W, and Godfrey, E Fed Proc, 7 226, 1948
9 Elliott, K A C, Scott, D B M, and Libet B J Biol Chem, 146 251, 1942
10 Elliott, K A C, and Henry M J Biol Chem, 163 361, 1946
11 LePage, G A, and Umbreit, W W in Manometric Techniques and Related Methods for the Study of Tissue Metabolism (W W Umbreit, R H Burris, and J F Stauffer, editors), Burgess Publishing Co, Minneapolis, 1945 p 159
12 Tobias, J M, Kollros J J, and Savit, J J Cell and Comp Physiol, 28 159, 1946
13 Jackson, D E Experimental Pharmacology and Materia Medica, C V Mosby Co, St Louis, 1939, p 523
14 Brauer, R W This Journal, 92 162, 1948
15 Koster, R This Journal, 88 39, 1946
16 Koelle, G B This Journal, 88 232, 1946
17 DuBois, K P, Erway, W F, and Byerrum, R U Fed Proc, 6 326, 1947
18 Beveridge, L Science, 93 356, 1941
19 Sawyer, C H, and Everett, J W Endocrinology, 39 307, 1946
20 Everett, J W, and Sawyer, C H Endocrinology, 39 323, 1946
21 Sawyer, C H, and Everett, J W Amer J Physiol, 148 675, 1947

THE EFFECT OF 2,3 DIMERCAPTO PROPANOL (BAL) ON THE TOXICITY OF 2-METHYL-1,4-NAPHTHOQUINONE TO MICE[1]

DAN A. RICHERT AND ALLAN D. BASS

Departments of Biochemistry and Pharmacology, Syracuse University College of Medicine

Received for publication October 2, 1948

The toxicity of 2-methyl-1,4-naphthoquinone in several species of animals has been established (1–3). The anti-microbial properties have also been demonstrated (4–9). Colwell and McCall (8) suggested that the mechanism of anti-bacterial and antifungal action of 2-methyl-1,4-naphthoquinone is dependent upon inhibition of —SH enzymes or metabolites essential for their growth. They reported that sodium thioglycolate, cysteine and ethyl mercaptan suppressed the anti-bacterial and antifungal action (effect) of the quinone.

Fieser (10) has demonstrated the interaction of 2-methyl-1,4-naphthoquinone and certain sulfhydryl containing compounds. And more recently Bartlett (11) has reviewed the evidence for a selective reaction between protein —SH and quinones.

Since BAL has been demonstrated to protect tissue —SH enzymes and since the evidence already quoted suggests that certain quinones produce their effects on biological systems through at least partial inactivation of —SH enzymes, we were led to study the effect of BAL on the toxicity of 2-methyl-1,4-naphthoquinone in a living mammal.

METHODS. Young adult Rockland white mice were used in these studies. No animals weighing under 19 grams were used. In general, 10 mice were selected as the minimum number to be used for any single procedure. The animals were fed Purina dog chow *ad libitum* and were allowed free access to water at all times. BAL (2,3 dimercapto propanol) was administered subcutaneously in saline in dosages shown in the accompanying tables. The volume used at no time exceeded 0.1 cc. The quinone was administered intraperitoneally in suspension in sesame oil in volumes not exceeding 0.2 cc. In those experiments in which the drugs were administered intraperitoneally in a single vehicle, sesame oil was employed. The 2-methyl-1,4-naphthoquinone was administered approximately five seconds after the BAL. Observations on the treated animals were made for a period of five days. The mortality results reported include animals dying within this period.

RESULTS. The results obtained are shown in table I. The data given demon strate the partially protective action of BAL in animals treated with 85 mgm. of 2-methyl-1,4-naphthoquinone per kilogram of body weight. That the converse also obtains is indicated by the results of July 23 (A) (table I). These data show that the quinone reduces the mortality resulting from highly toxic doses of BAL (100 mgm./kgm.). Table I [July 23 (B) and August 14] shows that there is a more marked antagonism between these two drugs when they are administered intraperitoneally in the same solvent.

[1] We wish to express our appreciation to Dr. D. W. MacCorquodale of the Abbott Laboratories for the 2-methyl-1,4-naphthoquinone used in this investigation.

TABLE I

DATE	MEDICATION	SOLVENT	ROUTE	DOSE	SEX	NO OF MICE	FIVE DAY MORTALITY
				mgm / kgm			
July 14	BAL	Saline	s c	40	Female	10	2
	Quinone*	Sesame oil	i p	75	Female	10	3
	BAL and Quinone	Saline Sesame oil	s c i p	40 75	Female	10	0
July 22	BAL	Saline	s c	50	Male	10	0
	Quinone	Sesame oil	i p	100	Male	10	10
	BAL and Quinone	Saline Sesame oil	s c i p	50 100	Male	10	9
July 22	BAL	Saline	s c	60	Male	10	0
	Quinone	Sesame oil	i p	85	Male	10	10
	BAL and Quinone	Saline Sesame oil	s c i p	60 85	Male	10	1
	BAL	Saline	s c	80	Male	10	1
	BAL and Quinone	Saline Sesame oil	s c i p	80 85	Male	10	4
July 23 (A)	BAL	Saline	s c	100	Female	10	8
	Quinone	Sesame oil	i p	85	Female	10	8
	BAL and Quinone	Saline Sesame oil	s c i p	100 85	Female	10	2
	BAL	Saline	s c	80	Female	10	5
	BAL and Quinone	Saline Sesame oil	s c i p	80 85	Female	10	3
July 23 (B)	BAL and Quinone	Sesame oil	i p	100 85	Female	10	0
August 14	Quinone	Sesame oil	i p	100	Male	5	5
	BAL and Quinone	Sesame oil	i p	100† 125† 150†	Male	10 10 10	0 3 5

* 2 methyl 1,4 naphthoquinone

† The BAL and Quinone were dissolved in sesame oil in equimolecular quantities The dose shown refers only to the dose of the 2 methyl 1 4 naphthoquinone

SUMMARY AND CONCLUSIONS

It has been demonstrated that BAL partially protects mice against lethal doses of 2-methyl-1,4-naphthoquinone and that greater protection is afforded when the two compounds are administered in the same medium. The results suggest that the affinity of the BAL molecule for the 2-methyl-1,4-naphthoquinone is not as great as the affinity of BAL reported for certain other chemical substances (12).

REFERENCES

1. Molitor, H., and Robinson, H. J.: Proc. Soc. Exper. Biol. & Med., *43: 125, 1940*.
2. Foster, R. H. K.: Proc. Soc. Exper. Biol. & Med., **45**: 412, 1940.
3. Smith, J. J., Ivy, A. C., and Foster, R. H. K.: J. Lab. Clin. Med., **28**: 1667, 1943.
4. Armstrong, W. D., Spink, W. W., and Kahnke, J.: Proc. Soc. Exper. Biol. & Med., **53**: 230, 1943.
5. Atkins, P., and Ward, J. L.: Brit. J. Exper. Path., **26**: 120, 1945.
6. Gonzalez, F.: Science, **101**: 494, 1945.
7. Colwell, C. A., and McCall, M.: Science, **101**: 592, 1945.
8. Colwell, C. A., and McCall, M.: J. Bacteriol., **51**: 659, 1946.
9. Geiger, W. B.: Arch. Biochem., **11**: 23, 1946.
10. Fieser, L. F.: Ann. Int. Med., **15**: 648, 1941.
11. Bartlett, G. R.: This Journal, **93**: 329, 1948.
12. Gilman, A., Philips, F. S., Allen, R. P., and Koelle, E. S.: This Journal, Supplement, **87**: 85, 1946.

PROLONGATION OF CURARIZING AND ANTI-CURARIZING ACTION[1]

HAROLD F CHASE, B K BHATTACHARYA, AND J L SCHMIDT

Departments of Surgery and Pharmacology of the University Hospitals, and Western Reserve University, Cleveland, Ohio

Received for publication October 2, 1948

Schlesinger (1) has reported on the use of a preparation of d-tubocurarine in peanut oil with 4 8 per cent beeswax (Romansky formula) (2) Favorable relaxant effects were observed in spastic individuals for 48 to 72 hours following intramuscular injections and some patients apparently received benefit from injections given as infrequently as once each week Similar residual beneficial effects were described earlier by Berman (3), following intravenous injection of a preparation of crude curare The present investigators considered that this beneficial effect might represent a special susceptibility of hyperstimulated muscles to the small residuum of curare present in the body, the characteristic effects of which otherwise might not be grossly recognizable It was apparent that the "head-drop" assay in rabbits, which has proved effective in testing anticurare action (4), might also be used to determine the amount of active curare remaining in animals at time intervals following injections of subparalyzant doses of curare

Because of the similarity of myasthenia gravis to the state of curarization, and the established sensitivity of myasthenic patients to curare, it was felt that the agents such as neostigmine, useful in the treatment of that disease, might be assayed in rabbits by observing their effects on "head-drop" doses of curarizing agents Since prolongation of neostigmine action would be most desirable in this disease, an attempt was made to delay the absorption of neostigmine by incorporating it in the beeswax and peanut oil vehicle This preparation was injected into rabbits and attempts were made to demonstrate its presence in physiologically active amounts by measuring the assayable sensitivity of the animals to curare The prolonged action of di-isopropyl fluorophosphate (DFP) following the intravenous injection of its aqueous solution was similarly studied

METHODS AND RESULTS The rabbit "head drop" assay, modified as previously described (4), was utilized in these experiments employing d tubocurarine chloride[2] as the standard agent for intravenous injection (as a solution containing 0 5 mgm /cc) d-Tubocurarine chloride was also available as a suspension containing 30 mgm (200 units)/cc in a peanut oil and 4 8 per cent beeswax vehicle[3] This suspension, when injected intramuscularly into the extensor muscles of the hind limb of rabbits, was absorbed erratically, as evidenced by the obvious curare effects in some animals and the paucity of observable or titratable effects in other animals at the same dose This variation

[1] This study was supported, in part, by a grant in aid from Hoffman La Roche, Inc

[2] Supplied through the courtesy of E R Squibb and Sons

[3] Supplied for these experiments by Abbott Laboratories

was presumed to be due to the differences in absorption and susceptibility due to variable activity of the animals (5). However, when injected beneath the dorsal skin in a dose of 4 mgm. (26.7 units)/kgm. there was little gross appearance of curare action to be observed and the rates of absorption and elimination found by assay of "head-drop" doses using the solution of crystalline d-tubocurarine were much more uniform. Twenty-four hours after subcutaneous injection of the oil-and-wax preparation into a group of 9 rabbits, the mean "head-drop" dose was 66 per cent of the control dose (critical ratio 4.4) and 48 hours after injection, it was 90 per cent of the control dose (critical ratio 2.5). These findings imply that at 24 hours, 33 per cent of a "head-drop" dose still remained.

An attempt was made to prolong the action of neostigmine methylsulfate, by suspending it in oil and wax in a concentration of 1.0 mgm./cc. Measurement of the effectiveness of this preparation was made by titrating the increase in "head-drop" dose of d-tubocurarine. The absorption of neostigmine was sufficiently rapid following subcutaneous injection of the suspension in doses of 0.75 mgm./kgm. that signs of parasympathetic stimulation (salivation, defecation, wheezing) were evident within 30 to 60 minutes. Eight hours after injection into 7 rabbits, the mean "head-drop" dose for the group was 106 per cent of the control value (critical ratio 1.52). A dose of 0.5 mgm./kgm. produced minimal effects and 4 hours post-injection the "head-drop" doses of the 7 rabbits averaged 119 per cent of the control dose (critical ratio 0.73). Neither result was considered to be statistically significant (critical ratio of 2.0 or greater), and the failure to prolong the action beyond 4 to 8 hours discouraged expansion of the series.

Control studies were made to determine the duration of anticurare effect of intravenously and subcutaneously administered crystalline neostigmine methylsulfate in water solutions containing 0.5 mgm./cc. "Head-drop" determinations with d-tubocurarine were performed at intervals of 1 minute, 30 minutes and 1 hour, in separate studies, following injections of 0.05 mgm. of neostigmine methylsulfate per kgm. intravenously. Similarly a group of 10 rabbits was tested at 15 minutes, 1 hour and 2 hours following injections of 0.1 mgm. of neostigmine methylsulfate per kgm. subcutaneously. The results of these assays are presented in figure 1. The means of all these assays differed significantly from the control mean, with the exception of the value for 1 hour following intravenous administration (critical ratio 1.14). It is evident, therefore, that with the doses employed, the anticurare effects last for ½ to 1 hour following intravenous injection and for 1 to 2 hours following subcutaneous injection.

The ability of intravenously administered DFP to antagonize curare action has been reported (4, 6). Additional information concerning the duration of this effect is included here for comparison with the duration of neostigmine effect. DFP was freshly prepared, in solutions containing 1 mgm./cc., before injection of each group of animals. A single dose of 0.1 mgm./kgm. was given to each of 13 rabbits, 0.2 mgm./kgm. to two groups of 14 and 9 animals, respectively, and 0.3 mgm./kgm. to a group of 18 animals. The results of the titrations of "head-drop" doses of d-tubocurarine immediately following and at

intervals of several days after injection are presented graphically in figure 2. The maximum antagonism to curare occurred following a dose of 0.2 mgm./kgm. and the duration of this action for 12 days or more simulates what is known of the duration of DFP effect on serum cholinesterase and approaches that of red blood cell cholinesterase. With the largest dose, the antagonism which appeared immediately was followed by prolonged synergism with curare. Muscular weakness lasting 1 to 2 weeks, or longer, has been described by Modell *et al.* (7) n cats surviving doses of 10 mgm. of DFP per kgm., but partially protected in the

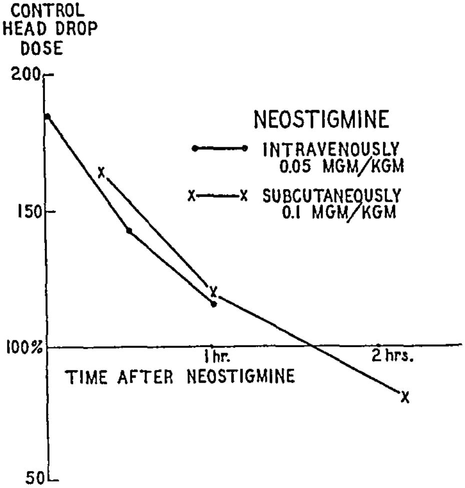


Fig. 1. Rabbit Head-drop Doses of d-Tubocurarine Following Neostigmine

acute stages of DFP poisoning by atropine, by atropine plus magnesium or by physostigmine.

Neostigmine or physostigmine could be given in repeated daily doses with impunity, but when rabbits, other than those previously described, were injected with DFP and d-tubocurarine for the second consecutive day, they presented many of the same signs as animals given a single fatal dose of DFP (0.6 mgm. per kgm.). During titration, convulsive and incoordinated movements were frequent, bronchospasm and salivation were marked, and the dosage required for the production of "head-drop," in nearly every instance, was lower than the one of the previous day. Recovery of ability to raise the head occurred quickly, but "head-drop" recurred intermittently for an hour or more thereafter in some animals. On subsequent days, following a total of 0.6 mgm. of DFP per kgm. in divided doses, the following phenomena were noted: (1) a number of animals

died; (2) in survivors which appeared to be relatively normal, the "head-drop" dose of d-tubocurarine was markedly reduced, usually to well below the original control level; (3) a total of 6 animals showed marked weakness of the neck muscles and were in a state of intermittent chronic "head-drop". These findings are similar to those recorded by Hunt and Riker (8) in cats chronically affected by DFP. Flaccid paralysis of neck muscles and fore-limbs following comparable amounts of DFP in rabbits has previously been noted by Horton *et al.* (9).

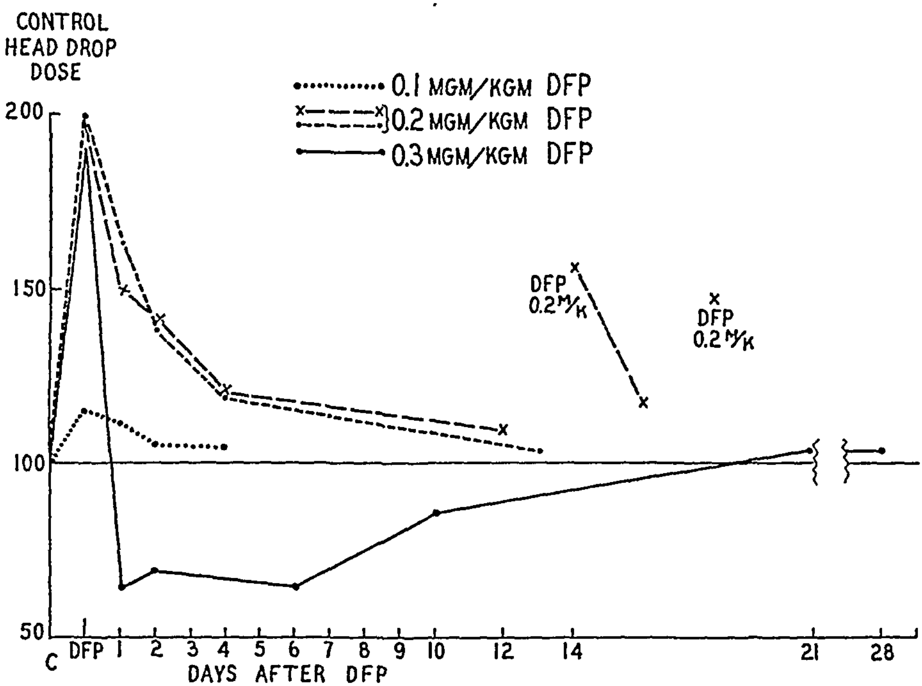


FIG. 2. RABBIT HEAD-DROP ASSAYS OF D-TUBOCURARINE FOLLOWING DI-ISOPROPYLFLUOROPHOSPHATE (DFP)

It is of considerable interest that in two of the animals subsequently showing markedly reduced "head-drop" doses of d-tubocurarine following DFP, a state of prolonged "head-drop" could be precipitated on the following day by neostigmine, 0.0125 mgm./kgm. In some of these rabbits, muscular exercise was also effective in inducing a return of "head-drop". We have previously demonstrated a synergism between the effects of exercise and curare action (5).

CONCLUSIONS

1. Measurable curare action has been demonstrated in rabbits by the rabbit "head-drop" assay method to be present 48 hours after the subcutaneous injection of a suspension of d-tubocurarine in a peanut oil and beeswax vehicle.

2. An assay method has been described by which the prolonged action on neuromuscular activity of preparations of neostigmine or other anti-cholinesterase substances can be measured.

3. Suspension of neostigmine in the peanut oil and beeswax vehicle failed significantly to prolong the action of neostigmine.

4. The duration of the antagonistic action toward curare of intravenously and subcutaneously injected neostigmine has been measured in rabbits; this corresponds closely with the duration of clinical effects of this drug seen in the treatment of myastenia gravis.

5. The duration of antagonism of curare action by single injections of DFP has been determined in rabbits.

6. The prolonged effect of large subcutaneous doses of neostigmine and large intravenous doses of DFP resulted in an increased sensitivity to curare.

REFERENCES

1. Schlesinger, E. B.: Bulletin New York Acad. Med., 22: 520, 1946.
2. Romansky, M. J., and Rittman, G. E.: Science, 100: 196, 1944.
3. Berman, M. S.: Arch. Neurol. and Psychiat., 41: 307, 1939.
4. Chase, H. F., Schmidt, J. L., and Bhattacharya, B. K.: This Journal, 91: 236, 1947.
5. Schmidt, J. L., and Chase, H. F.: Proc. Soc. Exper. Biol. and Med., 65: 13, 1947.
6. Chase, H. F., Schmidt, J. L., and Bhattacharya, B. K.: Fed. Proc., 6: 315, 1947.
7. Modell, W., and Krop, S.: This Journal, 88: 34, 1946.
8. Hunt, C. C., and Riker, W. F., Jr.: This Journal, 91: 298, 1947.
9. Horton, R. G., Koelle, G. B., McNamara, B. P., and Pratt, H. J.: This Journal, 87: 414, 1945.

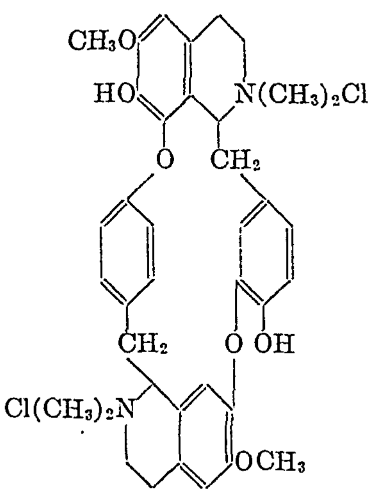
THE CURARIFORM ACTIVITY OF N-METHYLOXYACANTHINE

DAVID F. MARSH, D. A. HERRING AND CLARK K. SLEETH

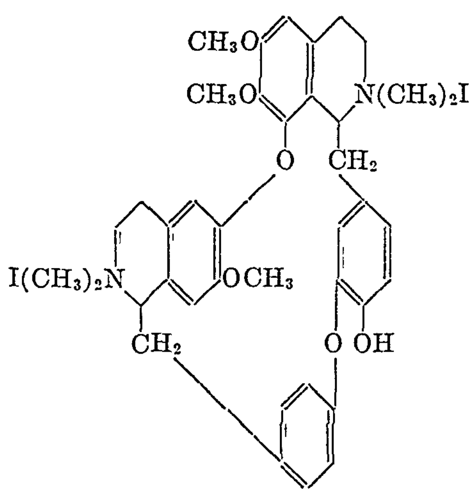
Departments of Pharmacology and Medicine, West Virginia University School of Medicine, Morgantown, West Virginia

Received for publication October 7, 1948

Oxyacanthine[1] was isolated in pure form from *Berberis vulgaris* by Hesse (1) and its structure elucidated by von Bruchhausen and Schultze (2). Examination of its formula reveals that this tertiary alkaloid has many structural components found in d-tubocurarine. We have converted oxyacanthine into its quaternary derivative, N-methyloxyacanthine iodide and have compared its pharmacological activity with that of d-tubocurarine chloride.[2]



I



II

I. d-Tubocurarine (3): Molecular weight 624.7. Used as the dichloride. Pentahydrate, molecular weight 785.7; m p. 268–269°C.
II. N-Methyloxyacanthine: Molecular weight 638.7. Used as the diiodide, molecular weight 892.6; m.p. 258–261°C.

Experimental Procedure. *Rabbits.* Solutions containing 0.25 mgm. of curariform ion per cc. were injected in 15 seconds into a marginal ear vein of each of 24 rabbits (1.8–2.4 kgm.); these were restrained individually in an enclosed box. The doses producing head-

[1] Also called hydroxyacanthine.

[2] This research was supported in part by a grant from S. B. Penick & Co., New York. We are grateful to Dr. W. G. Bywater, S. B. Penick & Co., New York, for generously supplying "western barberry" root from which the oxyacanthine was isolated by the method of Späth (Ber. 58: 2280, 1925). We are grateful to Dr. D. L. Tabern, Abbott Laboratories, North Chicago, for generously supplying the d-tubocurarine chloride, and to Dr. E. B. Tucker for technical assistance. After this research was completed, the preparation of N-methyloxyacanthine bromide was accomplished. With due allowance for the difference in molecular weight of the halide salts, this compound was identical in activity with the iodide.

drop lasting a minimum of 3 minutes in half a group of 8 animals were determined (see table 1). The experiments were repeated on the same animals after a one-week interval After an additional 4-day rest period, the 2-day head drop cross-over assay of Holaday (4) was performed. In this procedure, the rabbits are tied belly down and 0 1 cc. of solution administered intravenously every 15 seconds until the head will not rise when the shaven back is electrically stimulated. Since this is an assay requiring a positive response in all animals, the individual variation introduced is fairly wide; consequently, 0.15 mgm of d-tubocurarine chloride pentahydrate per kgm was considered one unit, and the data for the N-methyloxyacanthine was adjusted to this point, depending on the response of the individual rabbit on successive days. The results of the 24 pairs of data are given in table 1. Doses 50 and 100 per cent larger were given to each of 2 groups of 10 rabbits, and the LD_{50} was calculated by the method of Miller and Tainter (5).

TABLE 1

	D-TUBOCURARINE	N METHYLOXYACANTHINE
Albino rats		
LD_{50}	0.22 (0.27)*	0.43 (0.60)
Rabbits		
Head drop 50	0.10 (0 12)	0 13 (0.18)
Holaday head drop	0.12 (0.15)	0.15 (0.21)
LD_{50}	0.19 (0.24)	0.24 (0 33)
Dog		
Gastrocnemius muscle equivalent paralysis	0 06 (0 075)	0 11 (0 15)
Head drop	0.13 (0.16)	0.20 (0 28)
Man		
Head drop (3 minutes)	0.12 (0.15)	0.23 (0.32)
(9 minutes)	—	0.27 (0.375)
(18 minutes)	0 16 (0.20)	—

* All doses given in mgm of curariform ion per kgm body weight. Dose of equivalent amount of salt used given in parenthesis.

The animal data were treated by the method of Miller and Tainter (5). The standard errors of all the rat data lie within 6 per cent of the figures given, within 5 per cent for the rabbit data, and within approximately 9 per cent for the dog data In spite of the narrow limits of the calculated standard errors, there is a variation in these data for d-tubocurarine chloride and that of a previous publication (6).

Isolated sections of jejunum from 2 rabbits were prepared for recording by the usual Magnus technic in Tyrode solution and the responses to acetylcholine chloride and to histamine phosphate were tested before and after addition of the curariform agents

Rats The relative toxicity was determined in 60 albino male rats (170–250 grams). Solutions containing 0.10 mgm of curariform ion per cc. were injected intraperitoneally and the lethal doses were determined (see table 1). Four to 10 minutes after receiving a lethal dose, the animals became limp and unable to walk, respiration stopped in an additional 3 to 6 minutes, and finally cardiac activity ceased. The rats did not show any gross signs that these agents had cholinergic activity. They did not sneeze or salivate or evidence chromodachyria or flush as they do with some samples of crude curare.

Dogs. Since cats were unavailable, the gastrocnemius muscle preparation was performed on dogs. Three hundred mgm of sodium barbital per kgm. were administered intraperitoneally 90 minutes prior to operation in 6 dogs (5.7–8 4 kgm.). The femoral and sciatic

nerves to one leg were cut. The peripheral end of the cut sciatic nerve was stimulated for one-tenth second with 6 volts 60 cycles half wave every ten seconds by a motor driven interrupter. The contractions of the gastrocnemius muscle were recorded on a soot kymograph with a weighted lever. Carotid blood pressure was recorded with the usual mercury manometer. The dogs were mechanically oxygenated.

Fifty and 75 micrograms of d-tubocurarine chloride pentahydrate per kgm. were given intravenously as reference paralyzing doses. The dogs were apparently slightly less sensitive to d-tubocurarine than the cats used in a previous problem (6) since the larger dose was

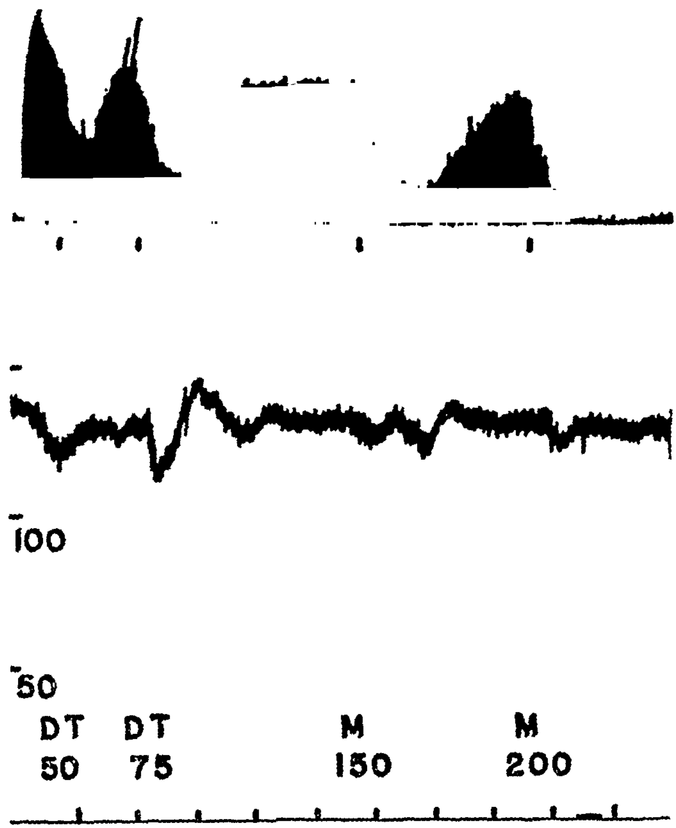


FIG. 1. 6.3 KGM. MALE DOG. GASTROCNEMIUS CONTRACTIONS, ABOVE, AND BLOOD PRESSURE, IN MM. HG, BELOW

Time marks at 5-minute intervals. Fifty micrograms d-tubocurarine chloride pentahydrate per kgm. given at DT/50; 75 micrograms at DT/75. One hundred fifty micrograms N-methyloxyacanthine iodide per kgm. given at M/150; 200 micrograms at M/200.

required to produce the expected amount of paralysis. After the muscle returned to normal, various doses of the other agents were given until equivalent paralysis was produced (see figure 1). The muscle could be partially paralyzed by this procedure for 4 to 8 times before contractility no longer returned to normal. Recent experiments indicate that the effects produced in a given animal by 65, 75, and 85 micrograms of d-tubocurarine chloride per kgm. differ by at least 5 per cent.

Four additional dogs were anesthetized with sodium baribital and were prepared for blood pressure recording. These animals received small doses of acetylcholine chloride and epinephrine hydrochloride before and after partially paralyzing doses of the curariform agents (see figure 2).

Modified head drop cross-over assays were performed in 3 trained, female litter-mate, pedigreed dogs (Boxers: 17–18 kgm., 10 months old). The agents (0.25 mgm of curariform ion per cc.) were administered intravenously in 30 seconds or less into the minor saphenous vein while the dog reclined on one side. The animal was allowed to rise, and the time of onset of paralysis (inability to stand) and its duration were recorded with any other objective findings. Doses of agents producing paralysis lasting 3–10 minutes could be readministered on the following day and the same duration of action, within ± 45 seconds, could be obtained. These assays with unanesthetized dogs were introduced as a preliminary to the investigations in man.

Man. In order to have information concerning possible clinical findings with N-methyloxyacanthine iodide, two experiments were carried out on one of us (D. F. M.) who had been previously standardized to d-tubocurarine chloride (6). The 80 kgm. 29-year old white

FIG. 2. 7.0 KGM. MALE DOG. 330 MGM. NA BARBITAL PER KGM.
micrograms)
AC; and 150
ve M. Rec-

male was placed in a modified Fowler's position on a treatment table (back elevated about 40° from the horizontal, legs about 120° to trunk). A strap was placed around the lower thighs to prevent the subject from slipping off the table. The N-methyloxyacanthine iodide was given intravenously over a two minute period as a 0.5 per cent solution. The outstanding features of the effects from one experiment are given in figure 3. Other than the occurrence of dryness of the mouth and nose, effects were similar to those noted with d-tubocurarine chloride (6).

RESULTS. Depending on the test procedure adopted, the N-methyloxyacanthine is one-half to three-fourths as active a paralyzing agent as d-tubocurarine chloride (based on molecular equivalence; slightly smaller ratios are obtained if the particular salts actually used are considered).

Although these two agents are primarily curariform in action, they differ slightly in side-effects. In our experiments with isolated rabbit jejunum, we found that as much as 800 mgm. of d-tubocurarine chloride per liter had no

apparent effect on the response of the gut to acetylcholine (1:30 million) or histamine, although 40 mgm. of N-methyloxyacanthine per liter blocked from 50 to 75 per cent of the effect of acetylcholine, but had little or no effect against the histamine. Similar effects were produced by 2 to 4 micrograms of atropine sulfate per liter. Similar lack of activity was found for d-tubocurarine chloride by McIntyre (7) although Gross and Cullen (8) found 'Intocostrin' to have atropine-like effects on human intestine.

Neither of these two agents has any obvious cholinergic or histaminic effects in intact rabbits or rats. In trained dogs, paralyzing doses of d-tubocurarine

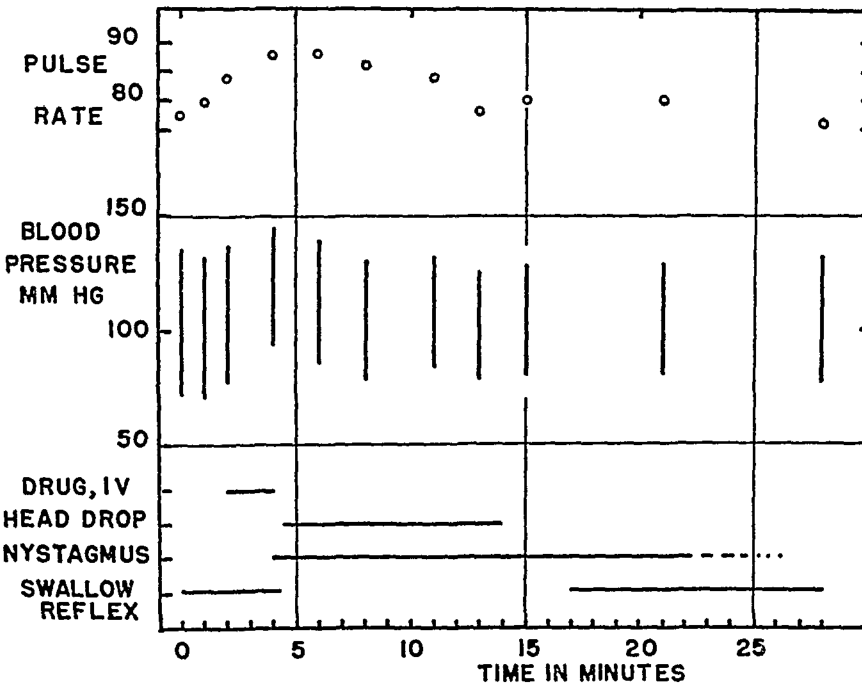


FIG. 3. HUMAN MALE (80 KGM.). THIRTY MGM. OF N-METHYLOXYACANTHINE IODIDE (0.5 PER CENT SOLUTION) WERE GIVEN INTRAVENOUSLY AT TIME INDICATED
Pulse rate, beats per minute, above; systolic and diastolic blood pressure, in mm. Hg, between; and presence of miscellaneous effects indicated by solid lines below.

chloride produce copious salivation; paralyzing doses of N-methyloxyacanthine iodide produce no salivation. A somewhat similar situation exists in man: the subject complained of the inability to swallow what he felt was the accumulation from normal salivation and the material had to be swabbed out by one of the operators periodically during the d-tubocurarine head-drop experiments; during the experiments with N-methyloxyacanthine the subject experienced a dryness of the mouth which persisted for several hours after the experiment had terminated and no swabbing was necessary..

In the anesthetized dog, d-tubocurarine chloride had no particular effects on the response to acetylcholine and epinephrine, although N-methyloxyacanthine

partially antagonized acetylcholine and slightly potentiated epinephrine (figure 2).

DISCUSSION. Brown and Fraser (9) discovered that almost any compound possessing a quinquevalent nitrogen atom within its molecule possessed some curariform activity. However, for a compound to be clinically useful as a curariform agent, either it must be specific enough that it does not produce any undesired side-effects or else it must combine two desired effects. Of the many quaternary ammonium compounds with curariform activity, only those with the d-tubocurarine type of molecule (I) have been sufficiently free of side-effects to be accepted clinically. Although d-tubocurarine is extensively used in co-anesthetic medication for relaxation of the abdominal musculature, it has not gained universal acceptance for use in convulsive and spastic states, whether these be the result of therapy (metrazol, electroshock) or disease and accidents. The introduction of an agent, such as N-methyloxyacanthine, which produces the desired skeletal muscle relaxant effect and yet does not produce the danger of laryngospasm from aspirated saliva, might lead to greater adoption of this type of therapeutic agent.

In an earlier paper (6), it was demonstrated that quaternary ammonium compounds which possessed the typical bis-1-(p-oxybenzyl)-6,7-dialkoxytetrahydroisoquinoline molecule had curariform activity without appreciable side activity. N-Methyloxyacanthine has virtually the same structural components except that they have been combined with the isoquinoline rings adjacent (II) instead of being alternated with the two oxybenzyl groups as in the d-tubocurarine molecule (I). This interchange of two rings has led to a slight reduction in activity (although several agents with the other arrangement are even less active (6)) and has added a weak atropine-like activity to the molecule.

SUMMARY

N-Methyloxyacanthine iodide was compared with d-tubocurarine chloride pentahydrate in rats, rabbits, dogs, and man. N-Methyloxyacanthine is about one-half as active a paralyzing agent as d-tubocurarine, but differs in that it also possesses a weak atropine-like action.

REFERENCES

1. HESSE, O.: Ber deut. chem. Ges., 19: 3190, 1886.
2. VON BRUCHHAUSEN, F., AND SCHULTZE, H.: Arch. Pharm., 267: 617, 1929.
3. KING, H.: J. Chem. Soc., 1948: 265.
4. VARNEY, R. F., LINEGAR, C. R., AND HOLADAY, H. A.: Fed. Proc., 7: 261, 1948.
5. MILLER, L. C., AND TAINTER, M. L.: Proc. Soc. Exper. Biol. and Med., 57: 261, 1944.
6. MARSH, D. F., SLEETH, C. K., AND TUCKER, E. B.: THIS JOURNAL, 93: 109, 1948.
7. McINTYRE, A. R.: Curare, University of Chicago Press, 1947.
8. GROSS, E. C., AND CULLEN, S. C.: Anesthesiology, 6: 231, 1945.
9. BROWN, A. C., AND FRASER, T. R.: Proc. Roy. Soc. Edinburgh, 6: 557, 1869.

THE EFFECT OF ANESTHETICS ON THE UPTAKE OF RADIOACTIVE PHOSPHORUS BY HUMAN ERYTHROCYTES[1]

V. PERTZOFF AND C. L. GEMMILL

Department of Pharmacology, Medical School, University of Virginia, Charlottesville, Va.

Received for publication October 8, 1948

The uptake of phosphate by the red blood cells has been the subject of many investigations although only a few determinations have been made using radioactive tracers. The migration of phosphate between plasma and cells was studied by Halpern (1). Her conclusions were that at 3°C. the membrane is not permeable to inorganic phosphate, at 23°C. slowly, while at 37.5°C. the transfer is easily demonstrated. Variations in carbohydrate metabolism were induced *in vitro* by the addition of glucose and sodium fluoride, and the subsequent changes in the distribution of phosphate were then observed. Inorganic phosphate entered the cell during glycolysis but left the cell when glycolysis was completed even though its concentration was already higher in the serum than in the cells. Eiseman, Ott, Smith, and Winkler (2), using radioactive phosphorus, confirmed the observations of Halpern. They found that inorganic phosphorus is excluded from the cells at 7°C. and freely enters the cell at 38°C. They believe that some inorganic phosphate is converted into an organic form. The mechanism of entry of phosphate into tissue cells has been reviewed recently by Sacks (3). In view of these experiments and the interest in the effect of anesthetics on cellular processes, it was decided to use the transfer of radioactive phosphate from plasma into the red blood cell as a means of studying the effects of anesthetics on this enzymatic transfer mechanism.

Early in the investigation, it was found that considerable variations exist in the uptake of phosphate by various bloods. Even the same individual does not show the same degree of penetration from day to day. Therefore, this research was extended to cover a study of thirty individual blood donors. Some of these individuals were tested repeatedly from day to day under uniform and also varied conditions of food intake.

METHODS. Human blood was used in this investigation. In some preliminary experiments it was oxalated (200 mgms. of potassium oxalate per 100 cc. of blood), but the majority of experiments were carried out with citrated blood using the ACD[2] preservative. One-hundred cc. of blood thus contained 1.91×10^{-3} moles of citrates at pH 6.8–7.0 and 600 mgms. of dextrose. In these latter experiments bloods of individual donors were used. When drawn in the morning the blood was used immediately; when drawn in the afternoon it was kept at 4°C. overnight.

---

[1] This investigation was supported (in part) by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service. The authors are also indebted to Dr. Vincent W. Archer, Department of Roentgenology, Medical School, University of Virginia, for supplying sufficient funds to purchase some of the equipment used in this work.

[2] ACD preservatives: Trisodium citrate, 166.2 grams; citric acid, 59.50 grams; dextrose, 300 grams. Added to 5000 cc. distilled water. Filtered and 50 cc. added to each 500 cc. blood.

A ten cc specimen of blood was placed in a test tube (20 × 140 mm) If necessary, the drug, in the form of powder, was added and dissolved One cc of radioactive phosphate in 0 9 per cent NaCl solution was then added, the tubes closed and rotated at a rate of 90 rpm at 37°C Control experiments without the drug were carried out in all determinations After various periods of time specimens were taken, centrifuged, and the plasma used for determination of radioactivity Simultaneously, hematocrit readings were taken to trace any possible variation in blood cell volume Hematocrit readings were made after 30 minutes centrifugation at 1700 rpm

For the determination of the amount of radioactivity, the following technique was found to give reproducible results Standard aluminum dishes (supplied by one of the manufacturers) were flattened by means of an hydraulic press The dishes were washed carefully with alcohol and ether By using a rubber stamp with a metal ring as a guide, a ring of silicon grease was stamped in the middle of each dish Then a 0 2 cc sample of

TABLE I

The effect of mass and the amount of plasma on the determination of radioactivity in plasma (per 0.2 cc of plasma)

Ashing the samples

SAMPLE NO	MINUTES FOR 2048 COUNTS	
	Dry	Ash
1	1 45	1 45
2	1 67	1 70
3	1 75	1 76

Varying the amount of plasma

SAMPLE NO	PLASMA	COUNTS PER MINUTE	COUNTS PER 0 1 CC
	cc		
1	0 1	310	310
2	0 2	580	290
3	0 3	890	297
4	0 4	1130	283

plasma was placed in the middle of the ring and with the help of a fine copper wire and a trace of di octyl-sodium sulfo succinate the plasma was spread evenly within the ring The sample was dried at 80°C To determine the radioactivity of the solution added to the blood, 1 00 cc of radioactive phosphate was added to 10 cc of water, and 0 2 cc of this solution was used for the determination of the radioactivity of the original phosphate solution

All measurements of radioactivity were carried out by determining the time required for 2048 counts to take place This operation was repeated ten times In consequence, every experimental point was based on 20,480 counts The results were corrected for the background counts which were generally at the rate of 30 to 40 per minute The over all average experimental error was about ±1 5 per cent

Two factors may interfere with the validity of the measurements The first is self absorption the absorption of beta radiation by the sample itself This factor was tested by ashing of the plasma and also by increasing the volume and, therefore, the weight of the sample In table I are given the results of these experiments Examination of this table shows that ashing does not change the time necessary for 2048 counts The same is true, up to a certain limit, when the amount of plasma is varied Therefore, self absorption in these experiments is negligible

Another variable in these experiments is the possible effect of carbon dioxide and oxygen on the penetration of radioactive phosphate. In table II are given the results of an experiment in which the same blood was saturated with air and with a mixture of 5 per cent carbon dioxide and 95 per cent oxygen. Surprisingly, these various bloods showed identical counts within the experimental error. The rate of penetration of radioactive ions is not affected by these gases under the conditions of these experiments. Therefore, it was decided to carry out all the determinations in atmospheric air. It is of interest to note that the absence of oxygen does not seem to affect this rate of penetration (table II).

In order to obtain the magnitude of the exchange of radioactive phosphate between cells and plasma the following determinations were made: 1. the total radioactive phosphate added to blood; 2. the amount of radioactive phosphate in an aliquot part of plasma; and 3. the volume of plasma (hematocrit readings). The number of counts and, therefore, the

TABLE II

The effect of saturating citrated blood with various gases at 37°C.

TREATMENT	COUNTS PER MINUTE PER 0.2 CC. OF BLOOD:			
	After 1½ hrs.		After 3 hrs.	
	In plasma	In blood corpuscles	In plasma	In blood corpuscles
Saturated with air	1070	820	750	1000
Sat. 5% CO_2 : 95% O_2	1000	890	770	980
Saturated with N_2	1040	850	760	990
Saturated with N_2	1010	880	740	1010

concentration of radioactive phosphate in plasma or cells can easily be calculated from the following equations:

$$\text{Counts in 0.2 cc. plasma} \times \frac{\text{Per cent of plasma}}{100} = \text{Counts in plasma per 0.2 cc. of blood}$$

$$\text{Counts in plasma } \frac{\text{per 0.2 cc. of blood}}{\text{Counts in 0.2 cc. of blood}} \times 100 = \text{Per cent of P 32 in plasma}$$

$$100 - \text{Per cent of P 32 in plasma} = \text{Per cent of P 32 in blood cells}$$

RESULTS. Considerable variation from individual to individual was found in the rate of penetration of radioactive phosphate ions into the cells. The frequency distribution diagram of 27 blood donors is given in fig. 1. The spread of experimental points is symmetrical after 1 hour and is less after 3 to 4 hours. After 6 hours, the segregation of experimental points was much better (not reproduced on the graph). This is probably due to the fact that the system approached an equilibrium.

The variation between individual blood donors led to the study of the variation of the rate of penetration of P 32 in a single individual under controlled conditions. J. L., a healthy, athletic, young man was asked to eat an average lunch between noon and 1:00 p.m. At 3:00 p.m. 50 cc. of his blood were drawn. The operation was repeated daily for 6 days. The results are given in fig. 2. The blood of J. L. showed considerable variability, equal, if not greater, than the variability between individual blood donors. Particularly striking are the results of two experiments when in 1 hour practically 80 per cent of P 32 was

transferred into the cells. The only noticeable difference which the blood showed on those days was a slight milky-white appearance. In consequence, it was thought that an acceleration of penetration of P 32 may have been due to the presence of fats or lecithin.

In order to clear up that point, it was decided to restudy an individual donor. This time, R. G. was requested to abstain from breakfast on 2 days (the blood was drawn at 9:00 a.m. and used immediately). On 2 other days, he was asked to eat a copious breakfast (8:00 a.m.), drink 2 glasses of milk and eat a large amount of butter. At 10:30 a.m., 50 cc. of his blood were drawn and used immediately for experiments. The results of these four experiments are given

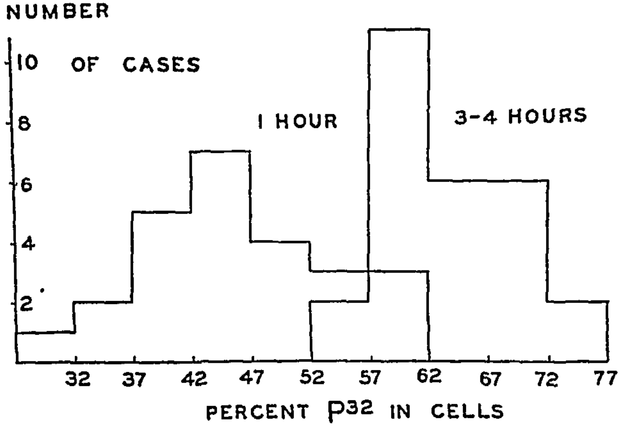


FIG. 1. RATE OF PENETRATION OF P 32 INTO HUMAN ERYTHROCYTES
Frequency distribution diagram of 27 individual blood donors. Citrated blood at 37°C. Abscissae, per cent P 32 in cells; ordinates, number of cases. Results are given for the determinations at the end of one hour and for the 3 to 4 hour determinations.

in fig. 3. Again this system demonstrated the same uncontrolled conditions. The food intake does not seem to be a governing factor in the rate of uptake of P 32. The two experiments before breakfast showed a wide gap at 1 hour. At 6 hours, the system showed a far more orderly behavior and the two sets of experiments exhibited a good segregation. The bloods before breakfast had about 60 per cent of P 32 in the cells; the bloods after breakfast, about 76 per cent.

The average curves for citrated blood (27 individuals) and for oxalated blood (10 experiments) are given in fig. 4. On the same graph is plotted the results of experiments with citrated blood as a reciprocal of time. This straight line was extrapolated to infinite time, indicating that at equilibrium conditions, 70 per cent of P 32 is in the cells.

The variability of the uptake of phosphate discussed in a previous paragraph

leaves two possible solutions: 1. to make an exhaustive study of a certain drug sufficient to give a probable quantitative solution of the problem, or 2. to make a restrictive study of the effect of a certain drug and be satisfied with the answer "yes" or "no." In the case of sodium barbital, an extensive investigation was carried out covering a large number of individual donors. In the case of ether, methadon, and urethane, only a few experiments were made to determine whether these drugs have an effect on the uptake of phosphate.

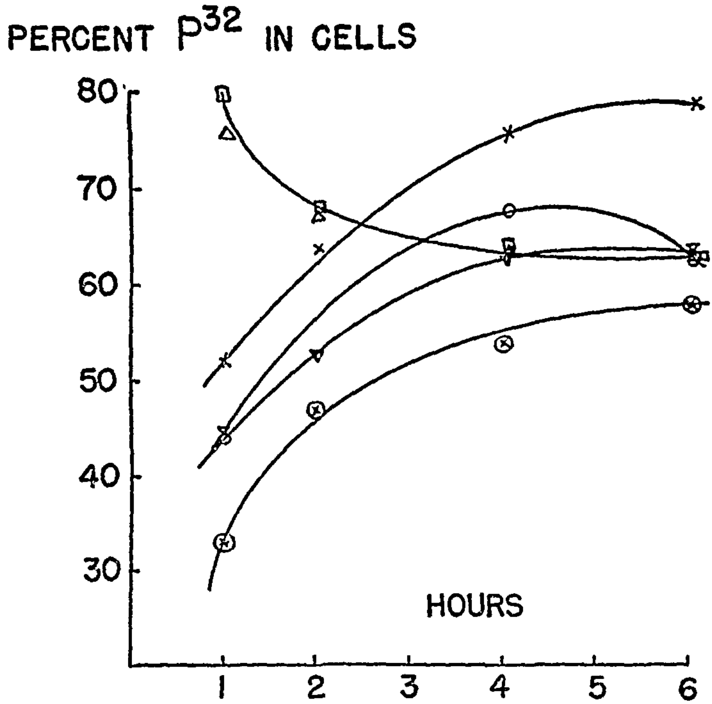


FIG. 2. RATE OF PENETRATION OF P 32 IN A SINGLE INDIVIDUAL (J. L.)
Six experiments on different days. Citrated blood at 37°C. Abscissae, hours; ordinates, per cent P 32 in cells.

Sodium barbital, in relatively small concentration, 0.02 Molar, has a distinct delaying effect on the uptake of radioactive phosphate by the cells. Fig. 5 gives the results of these determinations, using the bloods of 21 donors. In the same graph are found the corresponding controls to which no sodium barbital was added. This drug had a very definite effect at the beginning of the experiments, but after 6 hours the sodium barbital curve began to coincide with the control curve due to a higher rate of penetration of radioactive phosphate. It may, perhaps, be said that the mechanisms for uptake have the power of recovering from the initial inhibitory effect and gradually resume a higher state of migration.

Sodium barbital is osmotically active in blood, i.e., sodium barbital does not enter the cells. In table III are found the averages of determinations of the

change in volume of blood cells. Examination of the table demonstrates that the permeability of cells to sodium barbital does not change in the course of the experiment, indicating first, that the cell is not injured by a 6-hour contact with sodium barbital; and second, that the recovery of the cell from initial inhibition is not due to any redistribution of sodium barbital between the cells and plasma. Since sodium barbital is osmotically active, the blood cells lose water. Therefore, it may be suspected that the initial difference (1 hour) may simply be due to

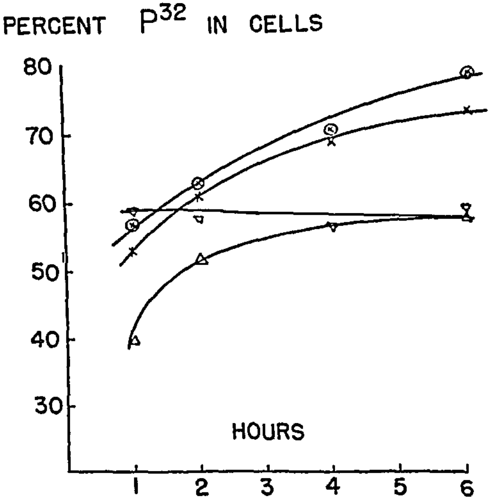


Fig. 3. Rate of Penetration of P 32 in a Single Individual (R. G.)
Citrated blood at 37°C. Abscissae, hours; ordinates, per cent P 32 in cells. Upper two curves, × and ⊗, blood samples taken after breakfast; lower two curves △ and ▽, blood samples taken before breakfast.

a flood of water from the cells into the plasma. This would produce a temporary physical barrier to the migration of radioactive phosphate into the cells.

If this delay is an osmotic phenomenon, then another osmotically active substance should reproduce the delay observed in the case of the sodium barbital curve. In table IV, the results of Exp. 50 are given in detail. In this experiment, sucrose was used as the osmotically active substance. The results do not leave any doubt concerning the specific effect of sodium barbital. In an iso-osmotic concentration, sucrose does not delay the penetration of radioactive phosphate into the cells. It must be concluded that the effect of sodium barbital is a true change in the mechanisms of uptake of phosphate. In order to produce the same amount of shrinkage in cells, the concentration of sucrose required is

Urethane was tried at concentrations of 0.02 and 0.04 Molar. The experiments extended from one to six hours. Beside the usual controls, experiments were done with urea at the same concentration as urethane. Urethane is osmotically inactive in blood; that is, it enters the cells. The results of four experiments indicate that neither urethane nor urea have any effect on the uptake of phosphate ions by the red blood cells.

DISCUSSION. In this study of the effects of certain anesthetics on cellular permeability, the system plasma-red blood cell was chosen on account of its ease of study. However, this simple system presented many difficulties in experimental analysis. Due to the variability in the rate of uptake of phosphate from day to day of blood from different donors and even from the same donor, each experiment had to be evaluated by itself. Fortunately, this could be done by using the same blood sample with and without the anesthetic present. Therefore, the blood sample in each determination had the same amount of citrate and glucose and was handled in the same manner, the only difference between samples being the presence or absence of the drug in question. There can be no doubt that sodium barbital in the concentrations used decreases the rate of uptake of radioactive phosphate by the red blood cell. Ether also has a retarding effect. Methadon (in very dilute solutions) and urethane do not effect the rate of uptake of phosphate by the red blood cell.

TABLE IV

Effect of sodium barbital and sucrose on cell volume and uptake of P 32 by red blood cells at 37°C. (citrated blood)

HOURS	CELLS BY VOLUME			P 32 IN CELLS		
	Control	0.04 *M* sucrose	0.02 *M* Na barb.	Control	0.04 *M* sucrose	0.02 *M* Na barb.
	%	%	%	%	%	%
1	37.9	34.9	34.7	50.9	52.6	39.0
3	38.2	34.7	35.1	61.5	62.6	52.7

From the fact that the rate of uptake of phosphate is abolished by a decrease in temperature and is dependent on the concentration of glucose in the plasma, it is obvious that the change in uptake is not merely one of change in permeability of the red cell membrane but depends on several factors. The chief factor must be an enzymatic action involving the formation of hexose-phosphates in the cell or on the cellular membrane. Therefore, it is probable that the anesthetics which delay the uptake of phosphate are acting on an enzymatic process.

SUMMARY

1. The rate of uptake of radioactive phosphate ions by human erythrocytes is a variable. It varies from individual to individual and in the same individual from day to day. This change seems not to be a function of food intake.

2. Sodium barbital decreases the rate of uptake of radioactive phosphate ions

by the red blood cell. Certain quantitative conclusions were derived from experiments covering a large number of individual blood donors.

3. Ether has a retarding effect. Methadon (in very dilute solution) and urethane do not change the rate of uptake of phosphate ions by the red blood cell.

REFERENCES

1. Halpern, L.: J. Biol. Chem., **114**: 747, 1936.
2. Eiseman, A. J., Ott, L., Smith, P. K., and Winkler, A. W.: J. Biol. Chem., **135**: 165, 1940.
3. Sacks, J.: Chem. Rev., **42**: 411, 1948.

Urethane was tried at concentrations of 0.02 and 0.04 Molar. The experiments extended from one to six hours. Beside the usual controls, experiments were done with urea at the same concentration as urethane. Urethane is osmotically inactive in blood; that is, it enters the cells. The results of four experiments indicate that neither urethane nor urea have any effect on the uptake of phosphate ions by the red blood cells.

DISCUSSION. In this study of the effects of certain anesthetics on cellular permeability, the system plasma-red blood cell was chosen on account of its ease of study. However, this simple system presented many difficulties in experimental analysis. Due to the variability in the rate of uptake of phosphate from day to day of blood from different donors and even from the same donor, each experiment had to be evaluated by itself. Fortunately, this could be done by using the same blood sample with and without the anesthetic present. Therefore, the blood sample in each determination had the same amount of citrate

TABLE IV

Effect of sodium barbital and sucrose on cell volume and uptake of P 32 by red blood cells at 37°C. (citrated blood)

HOURS	CELLS BY VOLUME			P 32 IN CELLS		
	Control	0.04 *M* sucrose	0.02 *M* Na barb.	Control	0.04 *M* sucrose	0.02 *M* Na barb.
	%	%	%	%	%	%
1	37.9	34.9	34.7	50.9	52.6	39.0
3	38.2	34.7	35.1	61.5	62.6	52.7

and glucose and was handled in the same manner, the only difference between samples being the presence or absence of the drug in question. There can be no doubt that sodium barbital in the concentrations used decreases the rate of uptake of radioactive phosphate by the red blood cell. Ether also has a retarding effect. Methadon (in very dilute solutions) and urethane do not effect the rate of uptake of phosphate by the red blood cell.

From the fact that the rate of uptake of phosphate is abolished by a decrease in temperature and is dependent on the concentration of glucose in the plasma, it is obvious that the change in uptake is not merely one of change in permeability of the red cell membrane but depends on several factors. The chief factor must be an enzymatic action involving the formation of hexose-phosphates in the cell or on the cellular membrane. Therefore, it is probable that the anesthetics which delay the uptake of phosphate are acting on an enzymatic process.

SUMMARY

1. The rate of uptake of radioactive phosphate ions by human erythrocytes is a variable. It varies from individual to individual and in the same individual from day to day. This change seems not to be a function of food intake.

2. Sodium barbital decreases the rate of uptake of radioactive phosphate ions

INHIBITION OF SUCCINIC OXIDASE SYSTEM BY MEPERIDINE,[1] METHADON,[2] MORPHINE AND CODEINE[3]

DANIEL T. WATTS

Department of Pharmacology, School of Medicine, University of Virginia, Charlottesville, Va

Received for publication October 13, 1948

Since the work of Quastel and his associates (1, 2, 3) there has been a growing concept that central nervous system depressants produce their effect by inhibiting the enzymes necessary for the oxidation of carbohydrates It has been shown that many narcotics inhibit oxygen uptake when glucose, lactate or pyruvate are used as substrate but do not inhibit the oxidation of succinate (3, 4, 5) These and other investigations (6, 7, 8) indicate the dehydrogenase, cytochrome and cytochrome oxidase are relatively narcotic insensitive, whereas flavoproteins or unknown components of the oxidative enzyme system are relatively narcotic sensitive Methadon, meperidine (demerol) and morphine, though very different in molecular structure, have similar *in vivo* action as analgesics A recent investigation (8) has shown that morphine in concentrations up to 0 01 *M* does not inhibit glucose oxidation by brain tissue *in vitro* whereas 0 005 to 0 01 *M* demerol and 0 001 to 0 005 *M* methadon do produce inhibition

The purpose of the present investigation was (a) to determine the effect of proven analgesics on the succinic oxidase system, (b) if possible to determine what part of the system was blocked and (c) to attempt to correlate *in vitro* and *in vivo* action of the drugs If the principal site of enzymatic blockage could be established then a biochemical basis would exist for the development of better analgesics and for the more efficient and economical testing of the large number of synthetic compounds being developed as possible analgesic agents Most recent investigations (9, 10) indicate the succinic oxidase system consists of the dehydrogenase, cytochrome b, an unknown factor, cytochrome c, cytochrome a and cytochrome oxidase Any observed effect of the analgesics on the oxidation of succinate would be due to its action on one or more of these components

METHODS The analgesics used were morphine sulfate USP, codeine sulfate USP, meperidine hydrochloride (Winthrop) and methadon hydrochloride (Lilly) Since there are two molecules of the active alkaloid per molecule of morphine sulfate and codeine sulfate, equal molar concentrations have twice as many active groups as methadon hydrochloride and meperidine hydrochloride

Beef brain homogenate was used as a source of enzymes About one hundred grams of brain, obtained within 10 minutes after the animal was slaughtered, were immediately

[1] Ethyl 1 methyl-4 phenylpiperidine-4 carboxylate hydrochloride kindly supplied by Winthrop Chemical Co as "Demerol"

[2] 4,4 Diphenyl 6 dimethylamino heptanone, 3 HCl kindly supplied by Eli Lilly & Co as "Dolophine"

[3] This investigation was supported in part by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health, U S Public Health Service

frozen in a mixture of dry ice and acetone. The frozen brain was then broken into pieces of one to three grams and packed in a 25 mm. x 200 mm. test tube. The test tube was slipped into a quart vacuum bottle, previously packed with about 500 grams of dry ice. When stored in a refrigerator the dry ice will last about one week; the tissue is kept at a very low temperature. There was no significant change in succinic oxidase activity for as long as 30 days. The tissue homogenate was prepared by grinding the frozen brain in cold 0.1 *M* phosphate buffer in a glass grinder (11). One part tissue plus 9 parts of buffer (10 per cent homogenate) was used for the Thunberg experiments; a 25 per cent homogenate was used for Warburg experiments.

Succinic oxidase activity was determined by the method of Schneider and Potter (12). Oxygen consumption was measured in Warburg manometers at 36.3°C. with a gas phase of oxygen. Two control vessels with no drug and four vessels with increasing drug concentrations were used in each experiment and served as checks on each other. The contents and concentration of the substances in the Warburg vessels are given with the results for each experimental condition. Oxygen was passed through the vessels 10 minutes followed by a 10 minute equilibration period. The initial readings were then made, the stopcocks closed and the substrate tipped over into the reaction vessels to start the experiment

The effect of the drugs on succinic dehydrogenase activity was determined by the rate of methylene blue reduction using the Thunberg method. The tubes and contents were chilled in an ice-water bath and evacuated for 3 minutes with a good water aspirator. Reduction time was determined visually using 90 per cent reduction as the end point. There was a tendency for the highest drug concentration, especially of morphine sulfate, to shift the pH of the buffered solution. A Cambridge glass electrode pH meter was used for pH determinations on the contents of the Thunberg tubes and the Warburg vessels after each experiment. Spectral absorption curves of cytochrome c were made with a Beckman model DU spectrophotometer. It could be determined qualitatively whether cytochrome c was in the oxidized or reduced state.

Results. The effect of the drugs on oxygen uptake by the succinic oxidase system is shown in figs. 1 and 2. Fig. 1 shows a typical experiment with meperidine. Oxygen uptake is linear with time and there is no significant endogenous activity of the homogenate. The relative inhibition of the succinic oxidase system by morphine, codeine, methadon, and meperidine is shown in fig. 2. The per cent inhibition was obtained by dividing the control vessel oxygen uptake for 60 minutes into the difference between the control vessel and the vessel with drugs. There is a marked inhibition by all the drugs. Part of the inhibition by morphine could be due to the shift in pH which was as much as 0.83 pH units at $4 \times 10^{-2} M$. The pH could not be held constant at 7.4 since morphine at the higher concentrations would not stay in solution at this pH. Methadon and meperidine gave an even greater inhibition at a very constant pH. The pH with these drugs never varied more than 0.1 units. Repeated experiments with methadon (fig. 2) and homogenates prepared at different times show the experiments and degree of inhibition to be quite constant and reproducible.

Spectral absorption curves made on the contents of the Warburg vessels immediately after experiments showed the cytrochrome c in the control vessels with a high rate of oxygen uptake was in the reduced state. This was to be expected since succinate in the presence of dehydrogenase readily reduces cytochrome c. In the vessels with concentrations of morphine, methadon or meperidine which produced significant inhibition, the cytochrome c was in the oxidized form. This indicated the dehydrogenase or some factor necessary for the reduction of cytochrome c by succinate was being blocked.

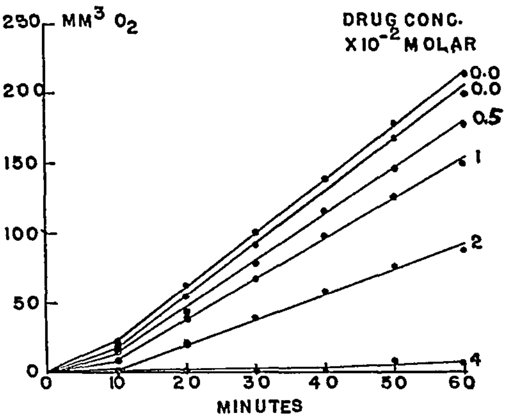


Fig. 1. The Effect of Varying Concentrations of Meperidine on the Succinic Oxidase System

hosphate
hrome c;
water to

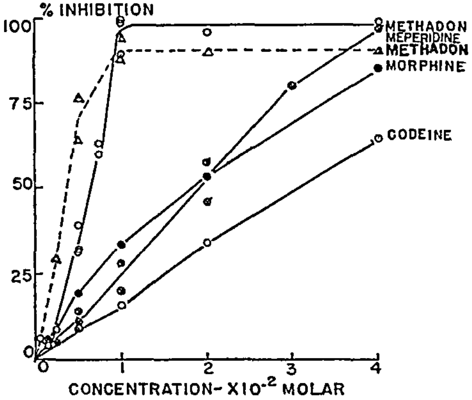


Fig. 2. Effect of Varying Concentrations of Analgesics on the Succinic Oxidase System

Solid lines, 0.3 cc. of 0.5 *M* sodium succinate as substrate; broken line, 0.3 cc. of 0.5 *M* *p*-phenylenediamine as substrate; concentration of other components same as for fig. 1.

Since succinic dehydrogenase catalyzes the reduction of methylene blue in the presence of succinate, it was thought this method could be used to determine

the effect of the drugs on the dehydrogenase. The results are given in table I. Morphine inhibited methylene blue reduction. This inhibition is partially due to the decrease in pH which amounted to 0.84 units at 1×10^{-4} M morphine sulfate. Time for methylene blue reduction increased from 7 to 27 minutes. In a control experiment when the pH was decreased 0.79 pH units by means of phosphate buffer the reduction time was increased from 18 to 25 minutes. Methadon and meperidine accelerated the reduction of methylene blue by the homogenate both in the presence and absence of succinate. This acceleration is independent of any change in pH which was never lowered more than 0.1 pH

TABLE 1

The effect of morphine, meperidine, and methadon on the rate of methylene blue reduction. Temp. 36.3° C.

DRUG	REDUCTION TIME—MINUTES				
Drug Concentration—Molar	0	0.005	0.01	0.02	0.04
Morphine sulfate	7	9	11	15	27
Morphine sulfate, no succinate	14	40	43	48	60
Meperidine HCl	30	26	25	25	18
Meperidine HCl, no succinate	101	71	39	23	12
Methadon HCl*	16	16	14	7	5
Methadon HCl* no succinate	49	34	7	1	1
Methadon HCl, no succinate, no homogenate	—	141	27	10	10

Except as noted each Thunberg tube contained 1.0 cc. of 1:10,000 methylene blue; 1.0 cc. of 0.10 M phosphate buffer, pH 7.4; 1.0 cc. of 0.15 M sodium succinate; drug to give final concentration shown above; water to make 5 cc.; 1.0 cc. of 10 per cent brain homogenate in phosphate buffer in the side arm.

* The methylene blue was in solution with the methadon during a 10 minute equilibration period so that some reduction had occurred before the homogenate was tipped into the tube and the data is only qualitative.

units. Finally it was found that methadon reduces methylene blue in the absence of both succinate and the homogenate. Thus methadon is a reducing agent which requires no biological catalyst to reduce methylene blue. Morphine and meperidine did not reduce methylene blue in the absence of the homogenate. Any observed effect of these analgesics on the rate of reduction would not necessarily be due to their effect on the dehydrogenase.

In a further attempt to isolate the point of blockage by methadon its effect on the oxidation of p-phenylenediamine was determined. Only cytochrome c—cytochrome oxidase is necessary for the oxidation of this substrate (9). As shown in fig. 2 methadon inhibits the oxidation of the p-phenylenediamine even more than the oxidation of succinate. Failure to obtain complete inhibition at the higher concentrations of methadon is probably due to the autoxidation of p-phenylenediamine.

Discussion. The analgesics investigated consistently inhibited the succinic oxidase system. The concentration required is about two to five times as great

as that required to inhibit the oxidation of glucose, lactate, and pyruvate by the same analgesics This is probably the reason most previous investigators have concluded that succinic oxidase is narcotic insensitive Assuming uniform distribution the concentrations required for *in vitro* inhibition are infinitely greater than that required to produce the *in vivo* effect of the drugs Though the relative analgesic potency of these drugs has not been firmly established (13, 14, 15), with the exception of meperidine the inhibition produced is in the order of the *in vivo* potency as analgesics

The attempt to determine the point of blockage by methadon or the other analgesics is inconclusive Any conclusions to the effect of the drugs on the dehydrogenase would be open to question since other factors effect the rate of methylene blue reduction The qualitative spectrographic data definitely indicated the dehydrogenase or an unknown factor (9, 10) is blocked, preventing the reduction of cytochrome c in the presence of succinate Since the oxidation of p-phenylenediamine is also inhibited it must be concluded that methadon also blocks at the cytochrome c—cytochrome oxidase level This might indicate that the inhibition is not specific but the analgesics at the concentrations used are general enzymatic inhibitors.

The author wishes to express his appreciation to Dr Chalmers L Gemmill for his many beneficial suggestions during the progress of this work and to Mrs R F Matthews for her technical aid in the experiments

SUMMARY

Morphine, codeine, meperidine, and methadon inhibit the oxidation of succinate by brain homogenate Spectrographic data showed these drugs prevent the reduction of cytochrome c by the enzyme preparation and succinate indicating the block occurred at the dehydrogenase or an unknown factor The inhibition of the oxidation of p-phenylenediamine by methadon shows blockage also occurs at the cytochrome-cytochrome oxidase level

REFERENCES

1 Michaelis, M , and Quastel, J H Biochem J , 35 518, 1941
2 Quastel, J H Physiol Rev , 19 135, 1939
3 Quastel, J H , and Wheatley, A H M Proc Roy Soc London, B112 60, 1932
4 Fuhrman, F A , and Field, J This Journal, 77: 392, 1943
5 Greig, M E This Journal, 87: 185, 1946
6 Jowett, M , and Quastel, J H Biochem J , 31: 565, 1937
7 Jowett, M , and Quastel, J H Biochem J , 31: 1101, 1937
8 Elliot, H W , Warrens, A E , and James, H P This Journal 91 98, 1947
9 Ball, E G , Anfinsen, C B , and Cooper, O J Biol Chem , 168 257 1947
10 Slater, E C Nature, 161 405, 1948
11 Potter, V R , and Elvehjem, C A J Biol Chem , 114 495, 1936
12 Schneider, W C , and Potter, V R J Biol Chem , 149 217, 1943
13 Scott, C C , and Chen, K K This Journal, 87 63, 1946
14 Scott, C C , Chen, K K , Kohlstaedt, K G , Robbins, E B , and Israel, F W This Journal, 91 147, 1947
15 Denton, J E , Straus, O H , Waddell, W E , and Beecher, H K Fed Proc , 7: 214, 1948

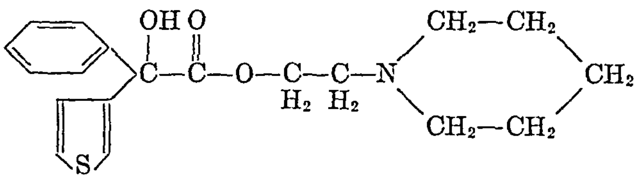
THE PHARMACOLOGY OF β-PIPERIDINOETHYL PHENYL-α-THIENYLGLYCOLATE HCl[1]

RAYMOND W. PICKERING, BENEDICT E. ABREU, JAMES Y. P. CHEN[2], RICHARD C. BURNETT AND WARREN C. BOSTICK

Divisions of Pharmacology and Experimental Therapeutics and Pathology, University of California Medical School, San Francisco, California

Received for publication October 13, 1948

A new series of anticholinergic spasmolytic compounds, substituted acetic acid and glycolic acid esters of amino alcohols synthesized by Blicke and Tsao (1), has been investigated recently by Lands and Nash (2) and Lands, Nash, and Hooper (3) for their pharmacologic activity. Preliminary experiments (4, 5) have shown that β-piperidinoethyl phenyl-α-thienylglycolate (PPT)



is most promising and worthy of more intensive pharmacologic study. The work described here was undertaken to determine chiefly the effectiveness of the compound with respect to its spasmolytic action in experimental animals and normal human volunteers, and to determine what side reactions might be expected to occur following therapeutic application to man.

METHODS. 1. *Animal studies.* a) *Cardiovascular action:* Healthy dogs of varying sex and weight were anesthetized with sodium pentobarbital. As an indicator of the anti cholinergic effectiveness of this agent, the blood pressure response to acetylcholine before and after the administration of PPT was determined. In order to determine whether PPT possessed any significant antihistaminic action, it was compared with diphenhydramine (Benadryl) employing histamine as the vaso-depressor substance. The intravenous route was employed throughout. Electrocardiographic studies were made in 4 unanesthetized monkeys after subcutaneous administration.

b) *Spasmolytic action:* Effects on the gastro-enteric tract were determined in 17 monkeys pretreated with morphine sulfate, 0.5 to 7.0 mgm./kgm., subcutaneously. The activity of the colon, ileum and jejunum in the acute experiments was measured after an abdominal incision and insertion of single balloons. In the remaining monkeys, the balloons were inserted in tandem into the distal colon by way of the anus and inflated to a pressure which would produce optimal recordings. Air or air-water transmission was used with either Harvard membrane or aneroid manometers. Intestinal activity of the dog was recorded similarly. Normal activity as well as morphine- and pilocarpine-induced hyperactivity were recorded. Intravenous dosages of morphine from 2.5 to 6.5 mgm./kgm. and of pilocarpine 2.0 mgm./kgm. were employed. Effects in the intact uterus were recorded in 4

[1] Aided by a research grant from the Sterling-Winthrop Therapeutic Institute, Rensselaer, New York, which also provided the new drugs used in this study.

[2] Now at National Institute of Health, Nanking, China.

anesthetized non pregnant multiparous rabbits, using the same recording apparatus as in the dog The activity of the isolated non pregnant, guinea pig strip was recorded by the Magnus method using Smith Ringer's solution Effects on the intact rat uterus were recorded in 5 anesthetized virgin rats PPT was administered subcutaneously in all cases

c) *Chronic toxicity* Three normal rabbits were given daily subcutaneous doses of PPT, 40 mgm /kgm until the animals died of convulsions During the period of this experiment, the sulfobromophthalein retention, blood urea, red and white blood counts, hemoglobin, and differential white cell count were determined every 10 days Particular attention was paid to the general condition of the animals, appetite cardiac rate, respiration size of the pupil and central nervous system irritability Autopsy was performed immediately after death and sections of the brain, lung heart, liver, spleen, kidney, adrenal, and intestine were taken for histopathologic examination

2 *Human studies* a) PPT was given to normal subjects in doses ranging from 2 5 to 35 mgm Doses of 2 5, 5, 7 5, 10, and 20 mgm were administered sublingually, those of 10, 15, 20 30 35 mgm , orally The following observations were made on each subject before and at definite intervals after the administration of PPT

Central nervous system Tremors, pupil size reading ability (as a measure of accommodation), degree of hypnosis (by the number of strokes tapped per minute on a telegraph key), degree of analgesia (by measuring the time of appearance of ischemic pain with a blood pressure cuff applied and pressure maintained over systolic blood pressure)

Autonomic nervous system Skin temperature color, and degree of perspiration

Cardiovascular system Pulse rate and character blood pressure and electrocardiogram

Respiratory system Rate and amplitude (by use of the metabolor)

b) Gastric motility was recorded in 4 subjects by means of a balloon passed into the stomach and connected by an air system to an aneroid manometer Doses of 5, 10, and 25 mgm were administered orally and of 5 and 10 mgm , sublingually

RESULTS—ANIMAL STUDIES *Cardiovascular effects* *Monkeys*—No effect was noted on cardiac rate, rhythm, or conduction in the monkeys after the subcutaneous administration of 4 0 mgm /kgm of PPT

Dogs—The fall in blood pressure from intravenous (i v) doses of acetylcholine iodide was effectively combatted by PPT in 14 dogs The mean fall in blood pressure of two dogs produced by a control dose of 0 001 mgm /kgm of acetylcholine iodide, i v , was 59 mm Hg After the administration of 10 0 mgm / kgm of PPT, i v , graded doses of 0 001, 0 01, 0 1, and 1 0 mgm /kgm of acetylcholine iodide produced a fall of 4, 16, 51, and 68 mm Hg, respectively It is interesting to note that after the administration of PPT, the fall in blood pressure caused by acetylcholine in dosage as large as 1 0 mgm /kgm (i e , 52 and 84 mm Hg) is comparable to that produced by the control dose of acetylcholine, 0 001 mgm /kgm

In view of the marked anti acetylcholine effect of PPT demonstrated by this experiment, more extensive studies were instituted in 5 other healthy dogs to determine the intensity and duration of action of this particular agent in smaller doses Following the injections in the same dog of decreasing doses of PPT, 10 0, 1 0, 0 5 and 0 1 mgm /kgm , i v , the control dose of acetylcholine, 0 001 mgm / kgm was repeated at 5 to 10 minute intervals until the blocking action of PPT against the depressor effect of acetylcholine could no longer be demonstrated Representative records of the decreased blood pressure response to acetylcholine at different intervals after the administration of PPT are illustrated in fig 1 The results show that PPT in doses of 0 1 mgm /kgm and up, possesses pro

nounced anti-acetylcholine activity. The depressor response to acetylcholine, 0.001 mgm./kgm., was completely blocked for considerably long periods by PPT in doses of 0.5, 1.0, and 10.0 mgm./kgm. and remained partially depressed for 71, 124, and 323 minutes, respectively.

In a series of 3 dogs, histamine was given in doses of 0.001, 0.01, and 0.1 mgm./kgm. No effect was evidenced on the histamine-induced fall in blood pressure

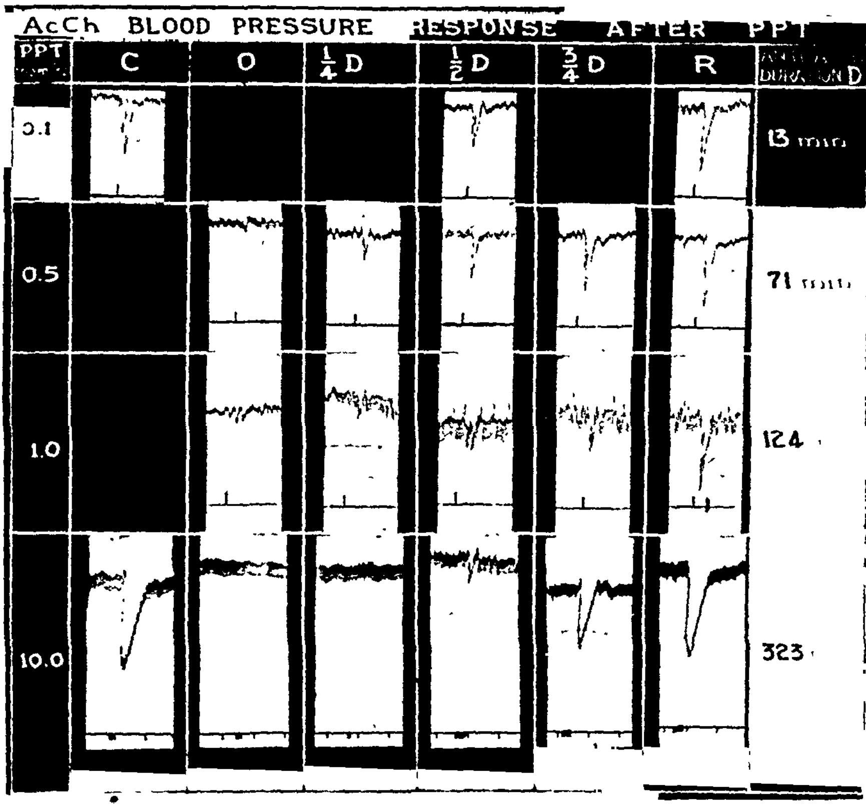


Fig. 1. Duratio ... effect of PPT on the blood pressure of the dog. Challenge dose of ... 001 mgm./kgm., intravenously, used throughout. C = control response to acetylcholine for PPT doses of 0.1 and 10.0 mgm./kgm. R = recovery response and control response for each subsequent injection of PPT; e.g. R for PPT 0.1 mgm./kgm. is control response for PPT 0.5 mgm./kgm., etc. D = total duration of anti-acetylcholine action expressed in minutes; ¼ D, ½ D and ¾ D represent responses to acetylcholine injections at time intervals which were ¼, ½ and ¾ of the total time (D) during which anti-acetylcholine action was present.

after the administration of 10 mgm./kgm. of PPT. There was no demonstrable effect upon the blood pressure rise induced by 0.1 unit/kgm. of pitressin after the intravenous administration of 10 mgm./kgm. of PPT.

Gastro intestinal activity. Monkey—Morphine was given to accomplish a dual purpose in the monkey experiments; first, to induce hypermotility, and second, to quiet the animal during the experimental period. No anti-morphine effect was obtained on the ileum, jejunum, and colon in an acute monkey experiment,

when PPT was administered in subcutaneous doses ranging from 0.1 to 0.4 mgm./kgm. No antispasmodic effect was found in the 7 remaining monkeys

TABLE 1

Duration of spasmolytic action of PPT and Trasentin, subcutaneously, following morphine induced hypermotility of the monkey colon

NUMBER OF MONKEYS	PPT	TRASENTIN	MEAN DURATION AND RANGE OF COMPLETE SPASMOLYSIS (MINUTES)
	mgm./kgm	*mgm./kgm*	
3	0.4	—	1 (0–3)
2	1.0	—	13 (5–21)
1	1.3	—	3 (1–6)
4	2.0	—	24 (8–52)
4	4.0	—	17 (5–28)
4	8.0	—	42 (5–62)
7		10	4 (0–22)

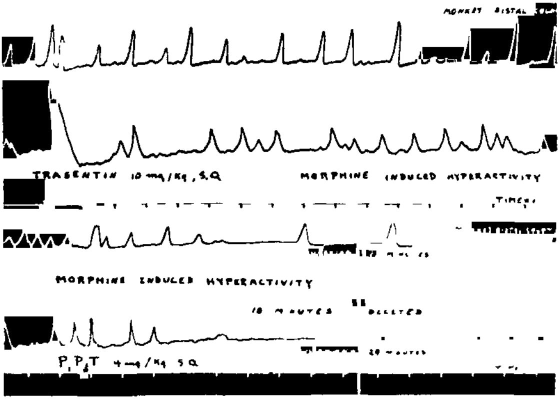


FIG. 2 INFLUENCE OF PPT AND TRASENTIN ON MORPHINE INDUCED HYPERACTIVITY OF THE MONKEY COLON

Upper record = tracing from upper balloon, lower record = tracing from lower balloon

with subcutaneous doses of less than 0.4 mgm./kgm. In the dosage range of 0.4 to 8.0 mgm./kgm. spasmolytic activity against morphine-induced hypermotility was usually quite marked, especially at the higher dose levels (see table 1).

A representative record is presented in figure 2. Trasentin did not produce spasmolysis until a dose of 10 mgm./kgm. was reached and then only infrequently did it exhibit any action on the monkey colon.

Dog—Results in normal, morphine- and pilocarpine-induced hyperactivity of the ileum and jejunum are summarized in table 2. PPT depressed activity in all of the dogs.

Uterine effects. Rats—No effect could be elicited on the intact rat uterus by PPT in subcutaneous doses ranging from 0.5–8.0 mgm./kgm.

Rabbits—No changes were observed in the intact uterus in doses varying from 1 to 10 mgm./kgm., given intravenously.

Isolated guinea pig uterus—In concentrations ranging from 5×10^{-4} to 5×10^{-2} mgm./cc., the activity was increased from 1 to 4 times that of the control.

Chronic Toxicity. The results of the chronic toxicity study are summarized in table 3.

TABLE 2

Spasmolytic effect of PPT in jejunum and ileum of the dog

NO. OF DOGS	PPT	RANGE AND AVERAGE DURATION OF DEPRESSION (MIN.)	AGENT USED TO INDUCE ACTIVITY
	mgm./kgm.		
1	0.2	0	None
1	0.5	34 (30–37)	None
1	2.5	101 (72–130)	None
1	0.5	40	Pilocarpine
1	1.0	58 (55–60)	Pilocarpine
3	0.5	21 (4–30)	Morphine
3	1.0	40 (0–130)	Morphine
1	2.0	52	Morphine

These tests were carried out over a period of 68 days. Slight changes in the blood picture of the tested animals were evidenced by an increase in the number of red and white blood cells.

There was slight impairment of renal and hepatic functions as evidenced by increase in blood urea and sulfobromophthalein retention. All of the animals showed a gradual decrease in body weight and gradual anorexia accompanied by general weakness. Definite mydriasis was regularly shown for about 3 hours following the injection from the second week of administration.

Rabbits No. 1 and No. 2 began to develop mild convulsions on the 44th and 47th day, respectively, and finally succumbed in clonic convulsions on the 46th and 52nd day, respectively. The third animal did not convulse until the 68th day when it died 2 hours after the onset of convulsions.

On autopsy 2 of these animals showed gross signs of congestion of the liver and scattered pale ischemic patches in the kidneys. Microscopic examination of

brain, lung, liver, spleen, heart, intestine, kidney and adrenal revealed no changes which could have been considered as being due to direct pathologic effects of PPT. In the case of one rabbit, early fatty infiltration ascribable to malnourishment was observed and in another there were recent intra-alveolar hemorrhages which could be explained as being due to the convulsive asphyxial type of death of these animals.

TABLE 3

Chronic toxicity in rabbits following the subcutaneous administration of PPT, 40 mgm./kgm. given daily

	RABBIT NO. 1			RABBIT NO 2			RABBIT NO 3		
	Control	2nd wk.	6th wk.	Control	2nd wk.	6th wk.	Control	2nd wk.	6th wk.
Body Wt. (kgm.)	3.5	3.8	3.0	4.1	4.3	2.9	3.2	3.0	2.5
Red Cell Count (millions)	5.57	6.32	6.27	6.13	5.37	6.84	4.81	6.71	6.93
White Cell Count (thousands)	12.2	14.2	17.7	9.7	11.9	14.8	11.5	18.3	21.7
Differential White Count									
N	75	66	59	71	70	64	69	62	68
L	19	31	36	22	23	33	22	34	26
M	4	3	5	7	6	3	8	4	6
E	2	0	0	0	1	0	1	0	0
B	0	0	0	0	0	0	0	0	0
Sulfobromophthalein % retention after 60 min.	17	18	24	10	12	15	20	22	27
Blood Urea N in mgm. %	10.4	12.0	13.6	12.5	14.0	14.7	9.5	12.5	14.0

TABLE 4

Untoward effects of PPT in humans

NO OF SUBJECTS	DOSE	ROUTE	SIDE EFFECTS
	mgm		
1	2.5	Sublingual	Local anesthesia at site of application
5	5	Sublingual	Local anesthesia at site of application
3	7.5	Sublingual	Local anesthesia at site of application
2	10	Sublingual	Local anesthesia at site of application
3	10	Oral	No effects
5	15	Oral	No effects
4	20	Oral	No effects
1	20	Sublingual	No effects; local anesthesia at site of application
4	25	Oral	No effects
4	30	Oral	Two subjects were drowsy
4	35	Oral	One subject was drowsy and another, 40 min. after administration, exhibited drowsiness, vertigo, and had dimness of vision and chills lasting 80 minutes

HUMAN STUDIES. Table 4 summarizes the results obtained in the determination of untoward effects in the human.

The average age of the subjects was 25, ranging from 17 to 35. As indicated in table 4, there were no appreciable effects noted until the 30 mgm. dose level was reached. Only one subject taking a 35 mgm. dose noted marked untoward effects; the first reactions noticed were dimness of vision and malar flushing 40 minutes after the administration of PPT orally; 35 minutes later, the subject had chills; her hands were cold, and forehead skin temperature had dropped 1.8° C. from the value obtained 15 minutes earlier. On standing, she complained that her legs felt "like rubber". No significant changes were noted in the pulse rate, blood pressure or electrocardiogram. The same subject had previously taken 15 mgm. with no untoward effects. Other than the effects indicated, there were no measurable changes observed by the methods employed on the central and autonomic nervous systems, cardio-vascular and respiratory systems.

TABLE 5

Inhibitory effects of PPT on gastric motility

DOSE	ROUTE	NO. OF SUBJECTS	ONSET OF ACTION	DURATION OF ACTION
mgm.			*min.*	*min.*
5	Oral	2	—	—
10	Oral	2	—	—
25	Oral	1	—	—
5	Sublingual	1	25	33
5	Sublingual	1	11	19
10	Sublingual	1	5	62
10	Sublingual	1	7	30

Table 5 illustrates the effects of PPT on gastric motility. Sublingual administration of 5 mgm. doses resulted in an inhibition of gastric motility for 19 and 33 minutes and 10 mgm. was spasmolytic for 30 and 62 minutes. Oral administration of 5, 10 and 25 mgm. doses did not inhibit gastric motility.

DISCUSSION. Investigations of agents which possess potent anticholinergic action indicate that when the desired antispasmodic effects are obtained, there will also be some action on effector organs other than those upon which action is desired. Side actions following the administration of a gastro-enteric antispasmodic to the normal human, may imply a host of anticholinergic effects. However, it should be recognized that a cholinergically innervated structure which is in spasm or hyperactive may be responding to a greater than normal amount of acetylcholine. Consequently, it would be reasonable to expect that depression of this type of activity would be possible without marked side effects. It is for this reason that most of the studies which have been conducted in this investigation have been designed to demonstrate drug effectiveness on a spasmogenic background.

PPT is apparently more effective in reducing the morphine-induced hyperactivity of the monkey colon than is trasentin. The preliminary experiments in

human volunteers indicate that PPT is effective in reducing the normal motility of the stomach and suggest that it deserves controlled clinical trial in diseases associated with hypermotility of the stomach and other portions of the gastroenteric tract.

In the monkeys used, there was no definite effect on the central nervous system in doses as high as 8 mgm./kgm. None of the animals exhibited noticeable side effects upon being released in their cages. However, it has been suggested (6) that the monkey is resistant to some of the peripheral and to the central nervous system effects of these anti-cholinergic compounds.

The central nervous system effects in man were definite only in one subject of four at the highest dose level of 35 mgm. orally. In all probability, this dose approximates the level at which untoward reactions will appear, since four other subjects receiving the slightly lower dose of 30 mgm. did not show any effect.

SUMMARY

1. *The cardiovascular effects of PPT are as follows:* (a) In monkeys, it produces no changes in cardiac rate, rhythm or conduction in subcutaneous doses of 4 mgm./kgm. (b) In dogs, it effectively abolishes the depressor effect of acetylcholine, but in doses as high as 10 mgm./kgm., intravenously, it does not alter the cardiovascular response to histamine or pitressin. (c) In the normal human, doses ranging from 2.5 to 35 mgm. orally, did not produce any changes in blood pressure, cardiac rate, rhythm or conduction.

2. *The gastro-enteric effects of PPT are:* (a) In monkeys, doses varying from 0.4 to 8 mgm./kgm., subcutaneously, induced spasmolysis in the intact colon rendered hyperactive by morphine. (b) In dogs, doses ranging from 1.0 to 2.5 mgm./kgm., intravenously, induced spasmolysis of normally active and hyperactive ileum and jejunum. (c) In the normal human, 5 and 10 mgm. doses, sublingually, depressed normal gastric motility.

3. *The uterine effects of PPT are:* (a) No changes were observed after intravenous doses ranging from 1 to 10 mgm./kgm. in the intact, non-pregnant, rabbit uterus or in the intact rat uterus with subcutaneous doses of 0.5–8.0 mgm./kgm. (b) Increase in activity was noted when concentrations ranging from 5×10^{-4} to 5×10^{-2} mgm./cc. bathed the isolated strip of the non-pregnant guinea pig.

4. *Chronic toxicity:* Daily administration of PPT to rabbits in doses of 40 mgm./kgm., subcutaneously, until death (46–68 days) slightly diminished renal and hepatic functions and increased red and white cell counts. The latter effect may be correlated with a progressive anorexia, weight loss, and dehydration. No significant histopathologic changes were produced.

5. *General effects of PPT in the human:* In doses from 2.5 to 30 mgm., there were no measurable changes in a two-hour observation period on central nervous, cardiovascular or respiratory systems. One subject, of four receiving 35 mgm., experienced vertigo, dimming of vision and chills lasting 80 minutes.

REFERENCES

1. Blicke, F. F., and Tsao, M. U.: J. Am. Chem. Soc., 66: 1645, 1944.
2. Lands, A. M., and Nash, V. L.: Proc. Soc. Exper. Biol. and Med., 57: 55, 1944.
3. Lands, A. M., Nash, V. L., and Hooper, K. Z.: This Journal, 86: 129, 1946.
4. Abreu, B. E., and Chen, Y. P.: Unpublished observations.
5. Lands, A. M.: personal communication.
6. Abreu, B. E., and Troescher-Elam, E.: This Journal, 86: 205, 1946.

THE ACUTE PHARMACOLOGY OF METHYL-*BIS*(2-CHLOROETHYL) AMINE (HN2)[1]

CARLTON C. HUNT AND FREDERICK S. PHILIPS

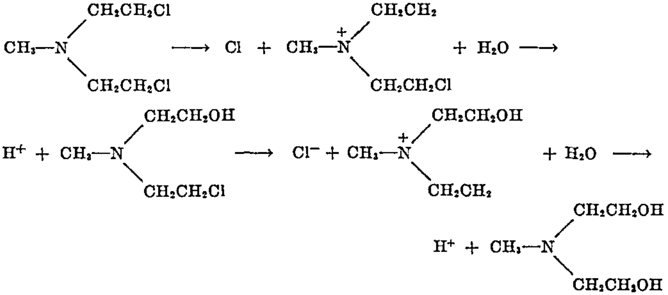
WITH THE TECHNICAL ASSISTANCE OF MARIE BORGATTA, JOSEPHINE HAWRYLKO, AND DORIS ROSENTHAL

Department of Pharmacology, Cornell University Medical College and the Pharmacology Section, Sloan-Kettering Institute for Cancer Research, New York

Received for publication October 15, 1948

Recent studies of *bis*(2-chloroethyl) substituted tertiary amines have emphasized their selective actions on proliferative tissues which resemble the effects of penetrating radiations (1). These cytotoxic actions have been used to advantage in the treatment of certain neoplastic diseases (2). The present study, however, concerns the acute pharmacology of methyl-*bis*(2-chloroethyl)amine (HN2) and its active transformation products. In this regard previous investigations of Gilman and Goodman (3) and Smith (4) have shown that HN2 has cholinergic actions, findings which were confirmed and extended by Foss and Gaddum (5). Evans and Foss (6) described the "neurotoxic" effect of certain transformations resulting from the reactions of HN2 in water. Gilman, Goodman, and Philips (7) investigated the pharmacology of products formed by the transformation of HN2 in buffered solutions and related certain of the pharmacologic effects of HN2 to the occurrence of such transformations *in vivo*. In addition, Anslow, Karnovsky, Jager, and Smith (8) investigated the toxicity of crystalline salts of a number of the transformed products of HN2.

METHODS. *Preparation of the transformed products of HN2.* The principal transformations of HN2 in dilute, slightly alkaline, aqueous solutions may be seen in the following series of consecutive reactions:



[1] The work described in this paper was done in part under contract between the Medical Division, Chemical Corps, U. S. Army, and Cornell University Medical College. Under terms of the contract, the Chemical Corps neither restricts nor is responsible for the

Only 3 of the principal stages in the transformation of HN2 have an appreciable existence in mildly alkaline solution, namely: methyl-2-chloroethyl-ethylenimonium (chlorimine), methyl-2-hydroxyethyl-ethylenimonium (hydroxyimine), and methyldiethanol-amine. The chlorhydrin formed by the hydrolysis of chlorimine undergoes rapid cyclization under such conditions. The cyclic chlorimine and hydroxyimine are involved, to a small extent in dilute solutions, in side reactions with different components of the mixture to form various relatively inactive polymers. Details of the reactions described have been fully studied by Bergman and co-workers (9). Advantage was taken of analytic procedures devised by these investigators to obtain solutions which contained as active constituents either the chlorimine or hydroxyimine. For this purpose 0.02*M* solutions of HN2-hydrochloride[2] were prepared in 0.16 *M* $NaHCO_3$ and maintained at 30 ± 0.1°C. for varying intervals. Analysis of aliquots for Cl^- liberated and $S_2O_3^=$ uptake permitted an evaluation of the extent of transformation at any given time.

Solutions obtained after 20 minutes of reaction were found suitable for study of the effects of chlorimine and showed upon analysis 2.00 molar equivalents of Cl^- liberated and 1.86 molar equivalents of $S_2O_3^=$ uptake. The analytic results indicate a complete transformation of the parent amine and the presence of chlorimine in solution in an amount equivalent to more than 90 per cent of the initial quantity of HN2·HCl. Chilling in ice water prevented any further significant change in composition for at least 2 hours. Doses of chlorimine were calculated assuming the 20-minute reaction product to contain 1 molar equivalent.

After 15.5 hours of reaction at 30°C., solutions showed no further liberation of Cl^- (2.87–2.91 molar equivalents). The 16.5 hour reaction product combined with 0.42 molar equivalents of $S_2O_3^=$. These results indicate the absence of components capable of cyclization and the presence of hydroxyimine equal to about 42 per cent of the initial amount of HN2·HCl. Chiling in ice water rendered this product stable for hours. Doses of hydroxyimine assumed the presence of 0.4 molar equivalents in such solutions.

After 48 hours of transformation, $S_2O_3^=$ reactivity was reduced to 0.03 molar equivalents. Such solutions were used to confirm previous observations on the relative inactivity of methyl-diethanol-amine and the various dimers formed during the transformation of HN2.

Analysis of transformation products. 1. *Chloride analysis.* Ten cc. aliquots were added to 5 cc. of 0.32 *M* acetic acid containing 5 drops of 0.1 per cent dichlorfluorescein solution. Titration for Cl^- was carried out rapidly with 0.1 *N* $AgNO_3$.

2. *Thiosulfate uptake.* Ten cc. aliquots were added to 10 cc. of 0.16 *M* $NaHCO_3$ and 10 cc. of 0.1 *N* $Na_2S_2O_3$. After reaction at room temperature for at least 2 hours, 10 cc. of 0.32 *M* acetic acid, 1 gram KI, and 1 cc. of 1 per cent starch solution were added in turn. Thiosulfate uptake was represented by the difference between the iodine (0.1 *N*) titer of the unknowns and that of 10 cc. of 0.1 *N* $S_2O_3^=$.

Cholinesterase activity. A modification of the method of Ammon (10) was used. The main wells of Warburg flasks received 3.0 cc. of 1:60 homogenates of salivary glands and 0.5 cc. of water. The side bulbs contained 0.5 cc. of 0.12 *M* acetylcholine bromide in 0.03 *M* $NaHCO_3$. Homogenates were made in the Waring blendor, the diluent being 0.03 *M* $NaHCO_3$. After gassing with 95 per cent N_2 and 5 per cent CO_2, temperature equilibrium was established at 38°C., the vessels tipped, and manometric readings taken every 10 minutes. A 30-minute period of constant rate was chosen to calculate activity.

Physiological methods. 1. *Blood pressure.* Adult cats were anesthetized with 30 mgm./kgm. of sodium pentobarbital intravenously and the carotid artery cannulated and con-

opinions or conclusions of the authors. The investigation was also supported by research grants to the Sloan-Kettering Institute from the U. S. Public Health Service, National Institute of Health, Division of Research Grants and Fellowships, and from the American Cancer Society.

[2] Twice recrystallized from hot acetone; analysis: N, 1.00 equivalent; total Cl, 2.97 equivalents; total $S_2O_3^=$ uptake, 1.99 equivalents.

nected to a mercury manometer. All injections were made into the femoral vein. The right vagus nerve was exposed for stimulation with an inductorium.

2. *Nictitating membrane.* The membrane of cats was connected to an isotonic lever after fixation of the eyeball. The cervical sympathetic truck was exposed for stimulation. Injections were made into the common carotid artery after appropriate dilutions were made with isotonic NaCl.

3. *Salivary flow.* Adult cats were anesthetized with 0.5 cc./kgm. of "Dial" solution (Ciba) intraperitoneally. The duct to the right submaxillary gland was cannulated and the salivary flow measured by a drop recorder. The *chorda tympani* was cut and there was no spontaneous flow. Injections were made into the right common carotid artery. In certain experiments the right superior cervical ganglion was removed. At the conclusion of these experiments the right submaxillary gland was removed for determination of cholinesterase activity.

TABLE 1

Intravenous toxicity of HN2·HCl and its transformation products in rats

DOSAGE	HN2·HCl		CHLORIMINE		HYDROXYIMINE		48 HOUR PRODUCT	
	Mortality	Time of death	Mortality	Time of death	Mortality	Time of death	Mortality	Time of death
mMols/kgm.								
0.40							0/6	
0.16	4/4	2 to 4 hrs.	6/6	3 to 4 hrs.	8/8	1 hr.		
0.08	4/4	1 day	6/6	1 day	9/16	1 to 3 hrs.		
0.04	4/4	1 to 2 days	6/6	1 to 5 days	1/6	3 hrs.		
0.02	4/4	3 to 5 days	6/6	3 to 6 days				
0.01	5/6	3 to 5 days	8/8	3 to 6 days				
0.005	6/14	3 to 6 days	2/10	4 to 5 days				
0.0025	1/10	7 days	0/6					

4. *Neuromuscular function.* Isometric recording of the contractions of the intact cat gastrocnemius-soleus preparation was made using a holder described by Wolff and Cattell (11). The tendon was wired to a heavy torsion lever, the popliteal artery exposed for injections, and the sciatic nerve cut and its distal end placed in enclosed silver electrodes. Supra-maximal nerve shocks were delivered by an interruptor from a 1 mfd condenser. The condenser charge was varied by a potentiometer circuit connected to a 45 volt battery. The frequency of stimulation was 1 per 12 seconds.

RESULTS. *Toxicity in rats.* The intravenous toxicity of HN2 and its transformed products was studied in Wistar rats weighing 100 to 250 grams. The toxicity and the survival times following administration of HN2 and its chlorimine were similar, (table 1). Doses less than 0.02 mM./kgm. caused delayed deaths while larger doses killed within 2 to 4 hours and were associated with a progressive muscular paralysis and terminal convulsive seizures. There was little difference in the appearance of rats receiving HN2·HCl and the chlorimine except that the latter sometimes produced a transient prostration immediately following the injection.

The hydroxyimine was less toxic but survival times following lethal doses were

shorter than those following equivalent doses of HN2·HCl or its chlorimine. This is in agreement with the studies of Boyland (12) who found that reduction in survival time of mice was related to the extent of hydrolysis of HN2. Rats which received the hydroxyimine in doses of 0.04 mM./kgm. or greater presented a progressive muscular paralysis which although more rapid in onset, was similar to that seen following HN2 or the chlorimine. Delayed deaths did not occur in animals receiving hydroxyimine. The 48 hour-product was relatively non-toxic and caused no deaths at 0.40 mM./kgm.

TABLE 2

Intravenous toxicity of HN2·HCl and its transformation products in cats

DRUG	DOSE	MORTALITY	TIME OF DEATH	FLACCID PARALYSIS	PARALYSIS + INCOORDINATION	REMARKS
	mM./kgm.					
HN2·HCl	.08	1/1	<18 hr.	0	+++	
	.04	1/1	<18 hr.	0	+++	
	.02	3/3	1d, 2d, 3d	0	+ −	
	.01	0/3		0	0	2 showed wt. loss
	.005	0/4		0	0	no signs
	.0025	0/2		0	0	no signs
Chlorimine	.02	3/3	15 min.			
			18 hr.	+++	+++	
	.01	3/3	<18 hr.	++	++	
	.005	3/4	1d, 6d, 14d*	0	+ −	
	.0025	0/2		0	0	no signs
Hydroxyimine	.02	3/3	31–50 min.	0	+++	
	.01	3/3	43–86 min.	0	+++	
	.005	3/4	55–75 min.	0	+++	
	.0025	2/6	43–48 min.	0	+++	
	.00125	0/2		0	++	recovery

* Sacrificed.

The results of the present study of the toxicity of HN2 and its transformations is in general agreement with the findings of Anslow, Karnovsky, Jager and Smith (8) who employed the corresponding crystalline salts.

Toxicity in cats. 1. *HN2·HCl.* The lowest intravenous dose of HN2·HCl which caused immediate effects in intact animals was 0.02 mM./kgm. (table 2). Within 10 minutes after such doses there appeared licking, retching, vomiting, salivation, and frequent, loose stools. Larger doses produced the above signs more rapidly and, in addition, a characteristic neurological disturbance which began within 20 to 30 minutes. Initially the disturbance was indicated by an inability to support the head; this was followed by a progressive paralysis of the entire somatic musculature. The advancing paralysis was associated with gross incoordination, asynergia, kinetic tremor, and dilated pupils which re-

sponded sluggishly to light. Coincidently there occurred profuse and sustained salivation. The paralysis became maximal within 1 to 2 hours after administration and, thereafter, the animal was prostrate making only abortive movements. Knee-jerks remained active throughout paralysis and no signs of muscular fasciculation or specific disturbance of the placing or righting reflexes was evident. Cats receiving 0.02 mM./kgm. were not paralyzed and succumbed after several days, following progressive weight loss. Convulsions were noted in only 1 cat on the day after administration of 0.02 mM./kgm.

2. *Chlorimine.* Chlorimine produced effects differing from those of HN2·HCl only insofar as its intravenous administration in doses of 0.01 mM./kgm. or more was followed immediately by complete paralysis. During the resulting prostration the skeletal musculature was flaccid and showed widespread fasciculations. Attending the initial paralysis was a brief period of salivation, pupillary dilatation, and bradycardia. Although respiration was stimulated during injection of the chlorimine larger doses were followed promptly by fatal respiratory paralysis. Cats surviving acute effects recovered rapidly and within 5 to 10 minutes walked about in apparently normal fashion.

Subsequently, at approximately 15 minutes after injection of 0.02 mM./kgm. or more of the chlorimine, there developed a second paralysis duplicating in detail that seen after injection of the hydrochloride salt. Inability to support the head was followed by ataxia, incoordination, asynergia, some hypermetria, and a progressive muscular weakness which reached a maximum in about 30 minutes. The delayed paralysis was associated with copious and prolonged salivation and pupillary dilatation. Although improvement of the neurological disturbance occurred, animals succumbed within 18 hours.

The effects of chlorimine differed from those of HN2·HCl principally in regard to the production by the former agent of two clearly separable paralytic disturbances. The chlorimine also caused less retching, vomiting, loose stools, blepharospasm, and ear flicking.

3. *Hydroxyimine.* Within a few minutes after the injection of hydroxyimine cats exhibited a neurologic disturbance which resembled in all respects the delayed paralysis and incoordination following HN2·HCl or its chlorimine. However, severe paralysis followed doses as small as 0.0025 mM./kgm. and consequently the agent proved to be more toxic than either HN2 or chlorimine. Maximal paralysis was obtained from 30 to 40 minutes after administration and was often associated with respiratory arrest. No skeletal muscle fasciculations were noted and salivation was scant in contrast to the copious flow which followed HN2·HCl or its chlorimine. Such animals were similar in appearance to those reported by Evans and Foss (6) after administration of a 24-hour, unbuffered, aqueous hydrolysate of HN2. These investigators in comparisons of the hydrolysate in monkeys, guinea pigs, rats, goats, dogs, cats and a horse found the cat to be most sensitive to its effects while the rat and guinea pig were relatively resistant. The hydrolysate consisted in large part of the chlorhydrin of HN2, the effects of which may be attributed to conversion *in vivo* to the hydroxyimine form.

Administration of hydroxyimine to cats also caused frequent, loose stools for several hours after injection. Cats which survived the paralytic effect of the hydroxyimine appeared normal on the following day and delayed deaths were not observed.

It is interesting to compare the acute actions of HN2 and its transformation products in cats with those noted by Gilman, Goodman, and Philips (7, 13). These workers observed parasympathomimetic effects in rabbits within 5 to 10 minutes after the injection of HN2·HCl which included salivation, defecation, lachrymation, bronchorrhea, and miosis. In addition skeletal muscle tremors and a rise in blood pressure followed intravenous administration in the atropinized cat. Such actions were attributed to muscarinic and nicotinic properties of HN2. It was also noted that chlorimine in rabbits was at least as toxic as the parent compound, evoked parasympathomimetic effects, caused immediate, transient, unexplained prostration, and produced delayed paralysis.

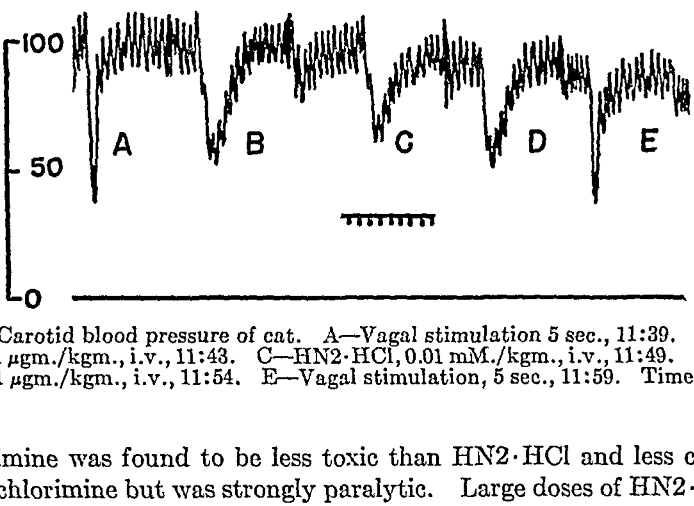


FIG. 1. Carotid blood pressure of cat. A—Vagal stimulation 5 sec., 11:39. B—Acetylcholine, 0.1 μgm./kgm., i.v., 11:43. C—HN2·HCl, 0.01 mM./kgm., i.v., 11:49. D—Acetylcholine, 0.1 μgm./kgm., i.v., 11:54. E—Vagal stimulation, 5 sec., 11:59. Time interval 10 seconds.

Hydroxyimine was found to be less toxic than HN2·HCl and less cholinergic than the chlorimine but was strongly paralytic. Large doses of HN2·HCl were shown to be convulsant in rabbits, an action not evoked by equivalent doses of chlorimine or hydroxyimine.

The present study is in agreement with the results obtained in the earlier observations mentioned above. However, in the cat miosis did not follow intravenous administration of HN2 or its transformed products and muscular fasciculations were evident only after injection of chlorimine. The coarse tremor which accompanied the delayed paralysis and incoordination involved whole muscle groups and was probably central in origin.

Effects on blood pressure. 1. *HN2·HCl.* Goodman and Gilman (3) and Foss and Gaddum (5) found that HN2·HCl caused a fall in blood pressure following intravenous administration, an action which was abolished by atropine and replaced by a rise after larger doses. Both groups considered HN2 to have muscarinic and nicotinic effects upon the circulation.

Figure 1 shows the fall in blood pressure following 0.01 mM./kgm. of HN2·HCl

intravenously The responses to vagal stimulation and to injected acetylcholine were not significantly altered by this dose Larger amounts (0 02 mM /kgm) abolished the circulatory response to vagal stimulation but had no significant effect on responses to injected acetylcholine or epinephrine The vagal blocking action of HN2 is less striking than that of *tris* 2 chloroethyl amine (14) No tachyphylaxis became evident after repeated doses of HN2

2 *Chlorimine* When given intravenously chlorimine caused responses similar to those following HN2 HCl but proved to be more potent than the parent amine Thus 0 002 mM /kgm was depressor while 0 01 mM /kgm caused pressor responses without the prior administration of atropine Figure 2 shows

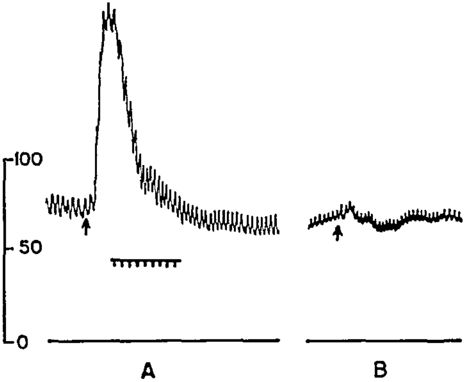


FIG 2 Carotid blood pressure of spinal cat Transection C 6–7 Received atropine, 2 mgm /kgm , i v A—Chlorimine, 0 01 mM /kgm i v , 11 05, Dibenamine HCl, 10 mgm /kgm , i v , 11 23 B—Chlorimine, 0 01 mM /kgm , i v , 11 40 Time interval 10 seconds

a rise in blood pressure following the intravenous injection of 0 01 mM /kgm of chlorimine in a cat which after a spinal transection at C 6–7 had received 2 mgm / kgm of atropine The administration of dibenamine, an adrenergic blocking agent (15), abolished the pressor response In view of these results and the fact that respiration was not depressed, the vasopressor action can be attributed to stimulation of sympathetic ganglia

Large doses of chlorimine abolished the circulatory response to vagal stimulation The agent also elicited transient bradycardia and varying degrees of heart-block as noted in electrocardiographic recordings The cardiac effects were prevented by prior administration of atropine

3 *Hydroxyimine* Intravenous injection of hydroxyimine caused cholinergic effects which resembled those elicited by HN2 or chlorimine However, larger doses were required for a given response Doses of 0 02 mM /kgm were depres-

sor in the absence of atropine but elevated blood pressure in cats previously treated with atropine (figure 3).

Nictitating membrane. Since the pressor effects of HN2, chlorimine, and hydroxyimine indicated stimulation of sympathetic ganglia by these agents, experiments were performed using responses of the cat nictitating membrane to

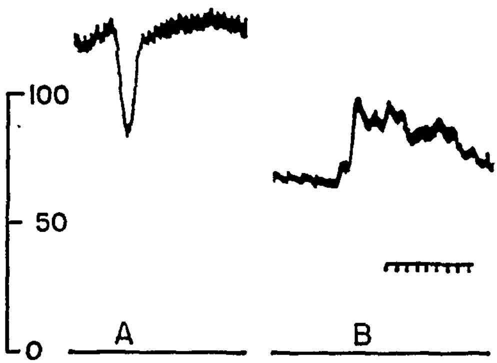


FIG. 3. Carotid blood pressure of cat. A—Hydroxyimine, 0.02 mM./kgm., i.v., 3:23; Atropine sulfate, 2 mgm./kgm , i.v., 3:57. B—Hydroximine, 0.02 mM./kgm., i.v., 4:00. Time interval 10 seconds.

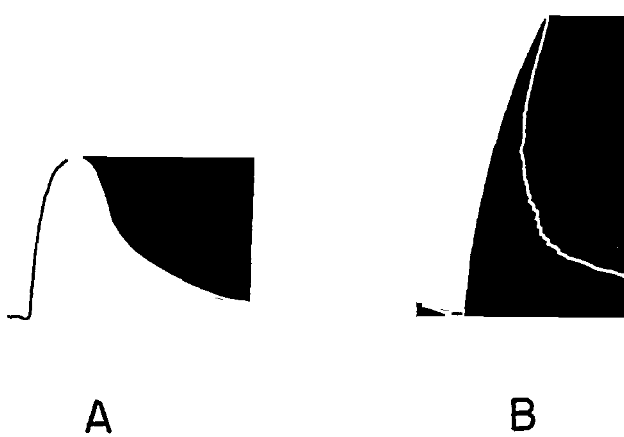


FIG. 4. Isotonic recording of nictitating membrane of cat. Received atropine, 1 mgm./kgm. A—Chlorimine, 0.02 mM./kgm., injected into common carotid artery of same side. B—Stimulation of cervical sympathetic trunk 2 minutes after A.

test their actions on the superior cervical ganglion. Figure 4 shows the contraction of the membrane resulting from injection of chlorimine in the common carotid artery. Crushing the superior cervical ganglion abolished this response. No ganglionic blocking effect of chlorimine was evident as judged by equal responses to preganglionic stimulation before and immediately after administration. While the effects of hydroxyimine were similar in this system, it proved to be less potent than chlorimine.

Effects on salivation Intravenous administration of HN2 or chlorimine in unanesthetized cats caused an immediate salivation of brief duration followed after an interval of 10 to 15 minutes by a second period of copious and prolonged flow In 1943 Foss and Gaddum (5) noted protracted salivation following intravenous injection of HN2 They also reported the prevention of this response by the prior administration of atropine and the fact that salivation could not be arrested, once initiated, by subsequent administration of atropine

To investigate this action, further experiments were performed on cats in which the duct of the submaxillary gland was cannulated Intra arterial injec-

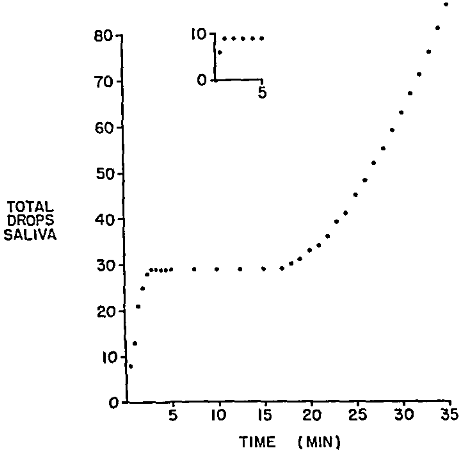


FIG 5 Salivary flow from right submaxillary gland of cat Right superior cervical ganglion removed At 0 time 0 02 mM /kgm of chlorimine injected into rt common carotid artery Note that there was no flow between 3 and 17 minutes after injection Insert shows typical response to the intra carotid injection of 10 μgm /kgm of acetylcholine

tion of HN2 evoked a unique and discontinuous response (figure 5) An immediate secretion of saliva was followed by a period of about 5 minutes during which there was no flow At the end of the inactive period salivation was resumed and gradually attained a maximal rate at which it continued for hours Salivary stimulation was, therefore, separable into two distinct phases the first of which resembled responses to injection of acetylcholine When atropine was administered prior to HN2 or chlorimine, salivation was completely prevented However, when atropine (2 mgm /kgm) was given by intracarotid injection after the initial appearance of salivation, even as early as in the interim between the two phases of salivation, it failed to alter the delayed response

Although HN2, in high concentrations, has been shown to inhibit cholines

terase *in vitro* (16, 17), this not the mechanism responsible for the protracted salivary stimulation. The submaxillary glands used in these experiments were tested for cholinesterase activity after the administration of HN2 and its transformed products *in vivo*. The mean activity of 17 experimental glands (102.5 cmm. CO_2/30 minutes) did not differ significantly from the controls (108.4 cmm. CO_2/30 minutes). Furthermore, atropine counteracts salivary response to anti-cholinesterase agents irrespective of the order in which it is given in contrast to the fact that it is ineffective when administered after HN2 or its chlorimine.

The unique action of HN2 and chlorimine on the salivary gland suggested the possibility that chemical alterations were involved in the diphasic response. Varying doses of the hydroxyimine were, therefore, injected in an attempt to reduplicate the prolonged salivary flow which followed HN2 or chlorimine. However, the response to hydroxyimine consisted only of a brief burst of saliva-

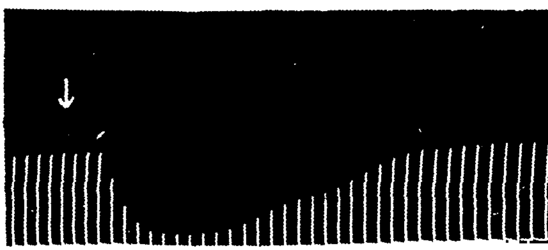


FIG. 6. Isometric recording of cat gastrocnemius-soleus preparation. Stimulation of sciatic nerve with maximal condensor discharges at frequency 1/12 seconds. At arrow intravenous injection of 0.02 mM./kgm. of chlorimine.

tion. Sustained flow was never observed. This response was also prevented by atropine.

Since HN2 and its transformed products caused stimulation of sympathetic ganglia, the possibility remained that at least some of the effects upon the salivary gland might be mediated through this mechanism. However, removal of the ipsilateral superior cervical ganglion did not alter the response of the submaxillary gland to HN2.

Paralytic effects. 1. *Chlorimine.* In order to investigate further the acute paralysis caused by chlorimine, experiments were performed on intact gastrocnemius-soleus preparations of the cat. Figure 6 is an example of the depressed response to maximal motor nerve stimuli which followed 0.02 mM./kgm. intravenously. Rapid recovery ensued and, thereafter, the contractile response remained normal for hours. During the period of severe depression of the response to indirect stimulation, the muscle still responded well to direct stimulation. At the same time the contractile response to close intra-arterial injection of acetylcholine was abolished. Recovery of responses to both acetylcholine

and motor nerve stimulation occurred simultaneously. When chlorimine was injected intraarterially, repetitive, asynchronous contractions could be demonstrated for 1 to 2 minutes before depression of the responses to indirect stimulation ensued.

While the transient depression of contraction can be related to the immediate prostration noted in intact cats receiving chlorimine, there was no effect upon the nerve-muscle preparation which could account for the delayed paralysis which follows the administration of either HN2 or chlorimine in intact animals. The above results are in accord with those of Brown (18) who noted a block in neuromuscular transmission in the frog nerve-sartorius preparation following exposure to HN2 or chlorimine. It is probable that the neuromuscular block which he observed following exposure to HN2 was due to its transformation to chlorimine in the organ bath. In the intact cat HN2·HCl failed to produce an immediate paralysis even following large doses. This may be ascribed to the failure of chlorimine formed *in vivo* from HN2 to attain paralytic concentrations in the circulation.

2. *Hydroxyimine.* In contrast to the immediate effect of chlorimine, even massive doses of hydroxyimine, given intravenously or intra-arterially, caused no depression of responses to maximal single motor nerve shocks or to tetanizing currents (frequency 60/sec.) of maximal intensity. Evans and Foss (6) noted that 24-hour unbuffered aqueous hydrolysates of HN2, which cause a paralytic disorder similar to that produced by hydroxyimine, had no effect on the contraction of the cat quadriceps following stimulation of the femoral nerve when intravenous doses as large as 40 mgm./kgm. had been given. In contrast, 0.5 mgm./kgm. caused definite paralysis in the intact animal. These investigators also found that 24-hour hydrolysates failed to depress spinal reflexes as judged by knee-jerks mechanically recorded in spinal cats. They did, however, note some decrease in the rigidity of decerebrate cats following administration of this agent. While the site and nature of the effect of hydroxyimine remains obscure, the paralysis is clearly not due to an action either at the neuromuscular junction or in the contractile mechanism of muscle.

Intestinal motility. HN2, chlorimine, and hydroxyimine caused stimulation of the isolated rabbit and guinea pig duodenum. The responses were prevented by adequate concentrations of atropine. Similar findings were reported by Foss and Gaddum (5) with rabbit duodenum and afford further evidence of the cholinergic effects of HN2 and its transformations. However, in the intact animal atropine had little effect on the diarrhea resultant from HN2 administration (5).

Discussion. Several groups of investigators have related the biological effects of nitrogen mustards to their known chemical transformations, (7, 8). HN2, as the prototype of this group of compounds may be considered to owe its varied and multiphasic pharmacologic effects to the production of a succession of such transformed products *in vivo.*

The acute pharmacology of HN2 is characterized by the cholinergic actions

of its two cyclic imonium transformation products and by a neurologic disorder produced by the latter of these products. Thus, the effects of the chlorimine of HN2 are manifested by cholinergic effectors of the autonomic nervous system, sympathetic ganglia, and skeletal muscle. In these loci the resultant stimulation or depression is a transient phenomenon, resembling the effects produced by certain choline esters. There is no evidence in the intact animal relating cholinergic actions to inhibition of cholinesterase. As noted by Foss and Gaddum (5) and confirmed in the present study, HN2 does not sensitize the animals to the circulatory effects of acetylcholine. However, Foss and Gaddum did find that doses of HN2, insufficient to stimulate the frog rectus preparation, sensitized the muscle to subsequent application of acetylcholine, an effect which they attributed to an eserine-like action. Nevertheless, the present study failed to reveal an inhibition of cholinesterase in submaxillary tissue of animals which had received doses of either HN2 or chlorimine sufficient to cause prolonged salivation. Furthermore, Feldberg (19) noted that concentrations of HN2 sufficient to reduce cholinesterase activity had an equally depressant effect upon acetylcholine synthesis.

As a cholinergic agent chlorimine is unique in causing a diphasic response of salivary tissue and in the failure of atropine to abolish salivation, once initiated. In contrast to other cholinergic effectors, salivary gland shows a strong affinity for chlorimine which could be conceived to undergo chemical transformation at this site and so evoke the secondary phase of salivary secretion. The latency between completion of the initial secretory response and initiation of the delayed phase might be due to the time required for such transformation to occur *in vivo*. The fact that the second response occurs coincidently with the onset of the delayed neurologic disorder in intact cats suggests this possibility. However, since protracted salivation cannot be duplicated by injection of hydroxyimine, it is difficult to eliminate the alternative possibility that the secondary phase of salivary stimulation represents a delayed response to an interaction with certain receptors which occurs at the time of the initial stimulation. In either case, the fact that prior administration of atropine prevents salivary response to chlorimine, suggests that the effects of the agent can be attributed to interactions with cholinergic receptors. However, it should be noted that Foss and Gaddum (5) attribute the delayed salivary response to a direct action of HN2 on glandular cells not mediated through receptor mechanisms. Obviously the mechanism of action of HN2 or chlorimine in salivary gland is obscure and warrants further study.

The paralytic actions of chlorimine also consist of an initial and a delayed phase. The former is clearly due to an action upon the neuromuscular junction, possibly related to the quarternary onium structure of chlorimine, while the latter is part of a complex neurologic disorder which is central in action. Thus, the two paralytic responses to chlorimine are related to different sites of action. Although the delayed neurologic disorder has not been localized, it may be defined as a disturbance of the motor system manifested by incoordination,

asynergia, progressive muscular weakness with loss of postural tone, and without apparent involvement of the vestibulo cerebellar system or of the spinal reflex arc Since animals which survive severe paralysis are normal on the following day, the disorder is probably functional rather than due to anatomic lesions

The delayed neurological abnormality which follows large doses of HN2 or chlorimine in the cat may be attributed to the production *in vivo* of hydroxyimine for the following reasons 1, it is manifested by administration of smaller doses of hydroxyimine than of HN2 or chlorimine, 2, a latency exists between administration of HN2 HCl or chlorimine and the production of the syndrome which may be explained by the time required for transformation to occur *in vivo*, and 3, the latent period before onset of the neurological disorder is significantly reduced by administration of hydroxyimine The greater potency of hydroxyimine in producing the disturbance may be explained by assuming that only a fraction of injected HN2 HCl or chlorimine is transformed to circulating hydroxyimine The neurological derangement is the most striking action of hydroxyimine and is probably its only significant contribution to the pharmacologic effects of even large amounts of HN2 HCl in the intact animal It is interesting to note a marked species difference between the cat and rat in susceptibility to this action

SUMMARY

1 The acute pharmacology of methyl-*bis*(2 chloroethyl)amine (HN2) has been investigated in rats and cats A standard procedure for transformation of HN2 was adopted which permitted the analysis of the effects of methyl-2-chloroethyl-ethyleniminonium (chlorimine), and methyl-2-hydroxyethyl-ethyleniminonium (hydroxyimine) The toxicity of these products by intravenous administration has been compared in cats and rats

2 The chlorimine of HN2 possesses striking cholinergic properties on effectors of the autonomic nervous system, sympathetic ganglia, and striated muscle It has a unique action upon the salivary gland producing a diphasic response which is prevented by the prior administration of atropine but which is not altered when atropine is given after secretion has begun

3 The hydroxyimine of HN2 is a less potent cholinergic agent and its principal effect is in the production of a neurologic disorder characterized by ataxia, incoordination, tremors, and muscular weakness

REFERENCES[3]

1 GILMAN, A, AND PHILIPS, F S SCIENCE, 103 409, 1946
2 "Approaches to tumor chemotherapy," pgs 285–378 Amer Assoc Advan Science, 1947, Washington, D C
3 GOODMAN, L, AND GILMAN, A 1942
4 SMITH, H W 1942
5 FOSS, G L, AND GADDUM, J H 1943
6 EVANS, C L, AND FOSS, G L 1942
7 GILMAN, A, GOODMAN, L, AND PHILIPS, F S 1942

[3] Where no reference is given, the material has not appeared in the open literature

8. Anslow, W. P. Jr., Karnovsky, D. A., Jager, B. V., and Smith, H. W.: This Journal, **91**: 224, 1947.
9. Golumbic, C., Fruton, J. S., and Bergman, M.: J. Org. Chem., **11**: 518, 1946.
10. Ammon, R.: Pflüg. Arch. ges. Physiol., **233**: 486, 1930.
11. Wolff, H. G., and Cattell, McK.: Arch. Neurol. and Psychiat., **32**: 81, 1934.
12. Boyland, E.: Brit. Jour. Pharmacol., **1**: 247, 1946.
13. Goodman, L., Gilman, A., and Philips, F. S.: 1943.
14. Cordier, D. G.: 1941.
15. Nickerson, M., and Goodman, L.: This Journal, **89**: 167, 1947.
16. Thompson, R. H. S.: 1942.
17. Barron, E. S. G., Barlett, G. R., and Miller, Z. B.: J. Exper. Med., **87**: 489, 1948.
18. Brown, D. E. S.: 1943.
19. Feldberg, W.: 1943.

NICOTINE IN BLOOD IN RELATION TO SMOKING[1]

WILLIAM A. WOLFF, MARINA A. HAWKINS, AND W. E. GILES

Tobacco Research Laboratory, Bowman Gray School of Medicine, Wake Forest College, Winston Salem, N. C.

Received for publication October 18, 1948

Information concerning the amount of nicotine in the blood of smokers is necessary for a study of the metabolism of this alkaloid in man. Quantitative data are available on nicotine absorbed from cigarette smoke in the respiratory tract (1). The quantity of nicotine excreted in the urine has been determined for smokers (2, 3). Other investigators have reported nicotine levels in milk from lactating women who smoked cigarettes (4, 5). Preliminary observations on nicotine levels in blood during smoking, reported elsewhere (6) have been repeated with a more trustworthy analytical method (7). The new data presented in this paper show that relatively little nicotine is present in the blood even after long periods of heavy smoking.

EXPERIMENTAL. All subjects in this study were healthy adults between the ages of twenty and thirty years, who, except for the slight inhaler group, habitually smoked twenty or more cigarettes daily. In order to keep the analytical "blank" (7) at a minimum value, the subject abstained from fish and from foods or beverages rich in purines for two days preceding the test. The subject smoked freely until late evening of the day preceding the test. On the morning of the test the initial blood sample was drawn by venipuncture before smoking began. During the next seven hours the subject smoked twenty standard brand cigarettes, about two thirds of each cigarette being smoked during a ten-minute period. Subjects were classified as deep, moderate, and slight inhalers. Cigars were smoked in one experiment and a pipe in another for a comparison with cigarettes.

Nicotine analyses (7). A distillate from alkalinized trichloroacetic acid filtrate of blood was treated with cyanogen bromide–beta naphthylamine reagents for estimating nicotine. The resulting color due to nicotine plus "blank material" was read in the spectrophotometer. A second aliquot of blood filtrate was treated with an activated carbon which selectively removed the nicotine. The distillate from this aliquot gave the amount of "blank material", and the difference between the two values was calculated as nicotine.

The range of nicotine levels in the blood before and after smoking cigarettes is shown for fifteen subjects in table 1. Two striking results are noted. The initial blood sample from each subject contained some nicotine *or a nicotine like substance* eight to ten hours after the last cigarette. Secondly, the nicotine level in the blood shows only a slight increase, from 0 to 0.13 mgm./l., over a seven hour period of heavy smoking. These data cannot be treated by statistical methods because of the small number of subjects in each group and the large overlap of nicotine values. However, there appears to be a significant difference between the deep inhalers and slight inhalers. The former show an aver-

[1] A preliminary report of this study was made before the American Society of Pharmacology and Experimental Therapeutics, Chicago Meeting, May, 1947.

This study was made possible by a grant from the Medical Relations Division, William Esty and Co., Inc., New York, N. Y.

age initial concentration of 0.20 mgm./l. of nicotine or nicotine-like substances, while the latter average 0.05 mgm./l. The deep inhalers tend to show a larger increase in blood nicotine on smoking than do the slight inhalers.

TABLE 1
Nicotine levels in blood

SUBJECT	NICOTINE IN BLOOD (MGM./L.)		
	Before smoking	After smoking	Increase
1 (D)*	0.12	0.25	0.13
2 (D)	0.16	0.18	0.02
3 (D)	0.35	0.43	0.08
4 (D)	0.18	0.26	0.08
5 (M)†	0.16	0.16	0
6 (M)	0.04	0.06	0.02
7 (M)	0.10	0.14	0.04
8 (M)	0.14	0.18	0.04
9 (M)	0.19	0.21	0.02
10 (M)	0.12	0.12	0
11 (M)	0.08	0.13	0.05
12 (M)	0.07	0.08	0.01
13 (S)‡	0.06	0.13	0.07
14 (S)	0.07	0.08	0.01
15 (S)	0.02	0.07	0.05

* D—Deep inhalation.
† M—Moderate inhalation.
‡ S—Slight inhalation.

TABLE 2
Nicotine levels in blood

SUBJECT	NICOTINE IN BLOOD (MGM./L.)		
	Before smoking	After smoking	Increase
4*	0.02	0.07	0.05
16*	0.12	0.20	0.08
17†	0.19	0.20	0.01
19†	0	0.02	0.02
20†	0	0.09	0.09
21†	0	0	0
13†	0	0.05	0.05

* Pipe smoked.
† Cigar smoked.

Nicotine levels in blood before and after seven hours of pipe or cigar smoking are shown in table 2. These subjects were cigarette smokers who changed to a pipe or to cigars for the test. Each subject tried to make his smoking with the pipe or cigar equivalent to smoking one pack of cigarettes in the same period. Although strict quantitative comparisons are not justified, it may be noted that

all the nicotine levels in this group fall in the same range found with cigarette smokers One subject, No 4, showed a blood nicotine increase of 0 08 mgm /l on smoking cigarettes and 0 05 on smoking a pipe Another subject, No 13, a slight inhaler, showed a rise of 0 07 mgm /l with cigarettes and 0 05 mgm /l with cigars These results suggest that blood nicotine levels show similar rises under the same conditions of smoking by the same subject whether a cigarette, cigar, or pipe is smoked

In an attempt to identify the material estimated as nicotine, filtrates from blood of smokers were concentrated by distillation and the final solution tested

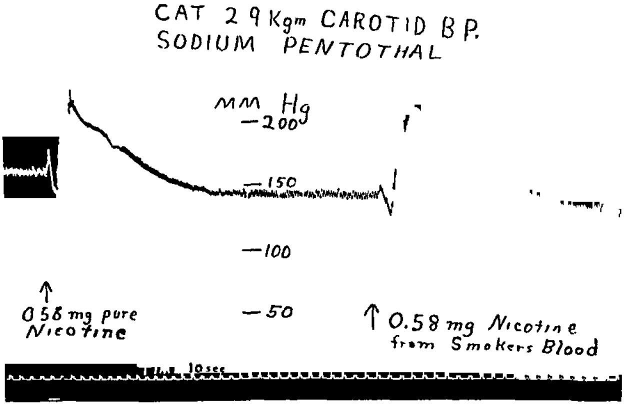


FIG 1 EFFECT ON BLOOD PRESSURE BY PURE NICOTINE AND BY NICOTINE ISOLATED FROM SMOKERS' BLOOD

for its effect on the blood pressure of a cat[2] The final concentrate, which represented nearly three liters of blood, was adjusted to pH 7 3 with hydrochloric acid before injection The blood pressure tracings, shown in fig 1, were made with a mercury manometer in the carotid artery of a cat anesthetised with sodium pentothal With the injection of 0 58 mgm of pure nicotine in saline solution, the blood pressure showed a small rise, a small drop, and a sudden rise of 80 mm mercury pressure, then a return to normal in about three minutes When 0 58 mgm nicotine isolated from the blood of smokers was injected into the same cat, the blood pressure showed essentially the same changes as with the pure nicotine, an initial rise, a small drop, and a sharp rise of 84 mm in pressure This result indicates that nicotine from smokers blood shows both

[2] Test made by Mr Russell Barnes

a qualitative and quantitative effect on blood pressure identical to that shown by pure nicotine.

COMMENTS. All the data presented here suggest a rather efficient mechanism for disposing of nicotine absorbed from tobacco smoke. It may be assumed that some 60 mgm. of nicotine is absorbed from the smoke of twenty cigarettes (1). Were this quantity of alkaloid equally distributed through all water in the body, its concentration would be 1.0 to 1.5 mgm./l. for subjects used in this study. Were the entire dose of nicotine retained in the blood stream, its concentration would exceed 10 mgm./l. The concentration of nicotine actually determined in blood at the end of the smoking period averaged 0.14 mgm./l. for twenty subjects. In view of the fact that nicotine appears to be freely diffusible across body membranes, we accept blood levels as an indication of the quantity of nicotine present in extravascular fluids or tissues. We may conclude that 80 to 95 per cent of the nicotine absorbed from smoke is metabolized during the period of smoking. A similar conclusion has been reached from studies on renal excretion of nicotine by smokers (2, 3).

The presence of nicotine in the initial blood sample drawn at the end of a period of eight or ten hours without smoking suggests that trace amounts of the alkaloid are metabolized or excreted very slowly. In contrast, there appears to be a much more rapid rate of metabolism with the larger doses of nicotine absorbed during smoking.

SUMMARY

Nicotine levels in the blood of smokers have been determined by a chemical method. Subjects who inhale deeply tend to show higher concentrations than do slight inhalers. Nicotine isolated from smokers' blood gave the expected effect on the blood pressure of a cat.

REFERENCES

1. PIERCE, I. H: J. Lab. and Clin. Med., **26**: 1322, 1941.
2. CORCORAN, A. C., HELMER, O. M., AND PAGE, I. H.: *J. Biol. Chem.*, **129**: 89, 1939.
3. HAAG, H. B., AND LARSON, P. S.: THIS JOURNAL, **76**: 235, 1942.
4. THOMPSON, W. B.: Am. J. Obst. and Gynec., **26**: 662, 1933.
5. PERLMAN, H. H., AND DANNENBERG, A. M.: J. A. M. A., **120**: 1003, 1942.
6. WOLFF, W. A., AND HAWKINS, M. A.· Fed. Proc., **6**: 386, 1947.
7. WOLFF, W. A., HAWKINS, M. A., AND GILES, W. E.: J. Biol. Chem., **175**: 825, 1948.

DIATROPINE DERIVATIVES AS PROOF THAT d-TUBOCURARINE IS A BLOCKING MOIETY CONTAINING TWIN ATROPINE-ACETYLCHOLINE PROSTHETIC GROUPS

K. K. KIMURA,[1] KLAUS UNNA, AND CARL C. PFEIFFER

Department of Pharmacology, University of Illinois College of Medicine, Chicago 12, Ill.

Received for publication October 18, 1948

Measurements on atomic models of acetylcholine and other drugs with specific muscarinic action have shown that these agents contain two or three oxygen prosthetic groups[2] at a distance of 5 to 9 A from one or more methyl on nitrogen prosthetic groups (1). Drugs which block the action of acetylcholine contain in addition to these prosthetic groups blocking or neutralizing moieties such as one or more butyl, or benzyl groups, or a benzohydryl group. Atropine is an extremely potent blocking agent for acetylcholine insofar as muscarinic effects are concerned. Its potency in blocking nicotinic effects of acetylcholine (on autonomic ganglia and on skeletal muscle) is extremely low. In contrast, d-tubocurarine blocks effectively the action of acetylcholine and other nicotinic agents on the skeletal muscle while it exerts little effect on autonomic ganglia, smooth muscle, or glandular cells. In an attempt to correlate spatial relationship of prosthetic groups to drug action, it was of interest to obtain inter-atomic measurements for the active groups of d-tubocurarine.

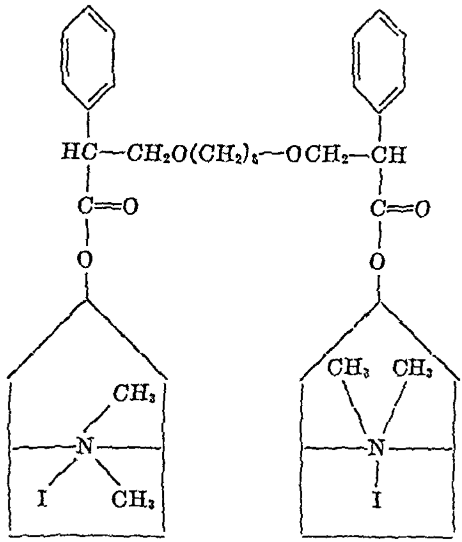
Using Hirschfelder atomic models the distance between the methyl on ring nitrogen and the three oxygen groups on the same hydrogenated quinoline ring of d-tubocurarine is within a radius of 5 to 9 A (this is in accord with the linear measurements between the prosthetic groups of acetylcholine). The two groups of oxygen atoms in d-tubocurarine average 9 A in their distance from each other and the nitrogens are 13 to 15 A apart. d-Tubocurarine, thus, resembles atropine in the "umbrella-structure" and the spatial arrangement of three oxygen groups to methyl on nitrogen. It differs from atropine in that it contains twin rows of these prosthetic groups instead of a single row. Hence, it would appear that the arrangement of twin rows of prosthetic groups is an important factor in endowing the molecule with its specific action on the neuromyal junction.

This hypothesis has been tested by a study of atropine and atropine-like homologues which have been joined together by a chain of approximately 9 A in length such as is provided by the normal amyl chain (Bovet *et al.* have shown that such amyl diethers have curare action [2]). In making amyl bis molecules of atropine and the quaternary salt of atropine two derivatives have been considered: 1) the dimethiodide of the amyl diether of atropine where two atropine molecules are joined through the tropic acid hydroxyl groups, and 2) the amyl di-quaternary compounds synthesized by connecting two atropine mole-

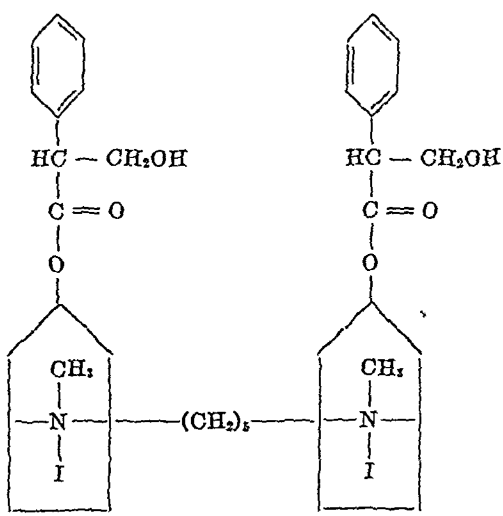
[1] Roche Fellow in Pharmacology.

[2] In acetylcholine, a methyl on carbon prosthetic group substitutes for the third oxygen prosthetic group while in neostigmine methyl on nitrogen substitutes for the third oxygen.

cules through the tertiary nitrogen with pentamethylene di-iodide (*figure* 1). Through the unfailing energies of Dr. L. C. Cheney of Bristol Laboratories we



O-Amyl-O-Diatropine Dimethiodide



N-Amyl-N-Diatropine Dimethiodide

FIG. 1. STRUCTURAL FORMULAE OF DIATROPINES

have been provided with these two compounds and similar "alkyl-bis" derivatives of other compounds with atropine-like activity. These are now being studied intensively for their possible practical value. Dibenadryl N-Amyl-N di-iodide was synthesized by *Dr. G. Rieveschl, Jr., of Parke, Davis* and *Company*.

The dibenadryl derivative was included because of the known atropine-like action of diphenhydramine (Benadryl) (3). That the findings are distinctly in accord with this hypothesis is shown by the data in table 1.

The compounds were studied for curare-like activity by standardized methods (4). The presence of block at the neuromyal junction was determined by muscular and sciatic nerve faradization in frogs following lymph sac injection. The lethal dose in frogs was

TABLE 1

Relative curare-like effect of atropine and diatropine derivatives

COMPOUND	MOL. WT.	MOUSE I.V.				RABBIT I.V. HEAD DROP	FROG I.L.	
		LD^*_{50}	Slope	HD_{50}	Slope		Peripheral paralysis	Death
Atropine sulfate.....	694	95.00 ± 6.2	b = 8.7			60†	1000	1000
Atropine Methyl Nitrate (Eumydrin)......	366	11.23 ± 0.89	b = 9.1	5.46 ± 0.44	b = 8.9	8.5	40	>200
Dibenadryl N-Amyl-N Diiodide.......	834	4.8 ± 0.24	b = 13			1.25	30	30
Diatropine N-Amyl-N Diiodide.......	902	1.23 ± 0.7	b = 12			0.350	30	200
Diatropine O-Amyl-O Dimethiodide......	929	0.79 ± 0.03	b = 19.3	0.58 ± 0.03	b = 12	0.325	7	20
d-Tubocurarine Chloride....	695	0.136 ± 0.003	b = 16.9	0.078 ± 0.002	b = 14.6	0.150	2	>10

* All doses are in mgm./kgm.

† This dose causes head drop followed by lethal convulsions.

determined in order to obtain some measurement of reversibility of the action on the neuromyal junction. The minimum dose causing head-drop (HD) in rabbits was determined by single intravenous infusions over a ninety-second period. Acute toxicity was studied in mice by intravenous injection at a rate of 0.1 cc. per 5 seconds of a suitable concentration of each drug so that a lethal dose was contained in 0.2 to 0.4 cc. The LD_{50} was calculated according to the method of Litchfield and Fertig (5). Mice succumbing to the various compounds apparently died of respiratory failure, in a manner similar to or identical with that observed with d-tubocurarine chloride. The margin of safety for the mouse was calculated by the ratio: LD_{50}/HD_{50}.

RESULTS. The curare-like action is markedly enhanced by the twinning of

quaternary atropine molecules through an amyl chain. Compared to atropine methylnitrate (eumydrin), both di-atropine compounds are about twenty-five times more effective when tested on rabbits, and about ten times more potent in mice. They show a less marked increase in potency in the frog. d-Tubocurarine chloride, however, is approximately 2 to 2.5 times more effective in rabbits than either di-atropine derivative.

Both di-atropine compounds exhibited a marked degree of specificity in their curarizing effects, as evidenced by the reversibility of their effects in frogs and rabbits. The ratio between curarizing dose (HD_{50}) and lethal dose (LD_{50}) for di-atropine O-amyl-O dimethiodide in mice is 1.36 compared to 1.74 for d-tubocurarine and 2.06 for eumydrin. The effects of the di-atropine compounds in rabbits were of short duration, and the recovery from paralyzing doses was materially aided by neostigmine.

The results furthermore stress the importance of one or more quaternary N atoms for curare-like action of these compounds. The curarizing effects of atropine in intact animals (frogs and rabbits) could only be demonstrated with doses which are convulsively or otherwise fatal to the animals (see table I). In contrast, the curarizing effects of eumydrin and the diatropines are reversible, and death in mammals is directly due to the paralyzing effect on the diaphragm. Furthermore, the non-quaternary diatropine O-amyl-O diether (not listed in the table) has indefinite curare-like action, when compared with diatropine O-amyl-O dimethiodide.

COMMENT. The marked increase in curare-like action by twinning prosthetic groups in a blocking molecule confirms our prosthetic group analysis of the d-tubocurarine molecule. The anatomical implications of this study in regard to the striated muscle cell are extremely interesting. Smooth muscle, glands and heart muscle have the acetylcholine effect blocked by atropine with its single row of prosthetic groups but striated muscle is much more effectively blocked by blocking molecules containing twin rows of prosthetic groups when the mean distance between the oxygen atoms is 9 A.

The recent studies of Barlow and Ing (6) and others (7, 8) on simple aliphatic di-quaternary diamines indicate that a chain length of C_{10} is optimal for curare-like action of the methylated diamines. These compounds are as potent as d-tubocurarine but are not antidoted by neostigmine. The diamines provide a distance of 15 A which is in agreement with our measurements on the d-tubocurarine molecule where the distance between the nitrogens is 13 to 15 A. The ideal arrangement may thus diagramatically be depicted as in figure 2. Thus the C_{10} chain should be used when the nitrogens are connected. Compounds with slight to moderate atropine action such as diphenhydramine have their toxicity increased and become curare-like when two molecules are connected by an amyl chain. This action should be more markedly enhanced if a C_{10} chain is used to link the two nitrogen atoms. Since the potent aliphatic diamines (6) contain no oxygen prosthetic groups and no blocking moieties (except the aliphatic chain), the careful study of N-C_{10}-N quaternary derivatives will decide between the relative importance of methyl on nitrogen prosthetic groups and the oxygen prosthetic groups.

These studies raise many interesting questions and speculations. Does the neuromyal junction of striated muscle have a front and back door of receptors, both of which must be blocked simultaneously? Does attachment of a single atropine or eumydrin molecule on the striated muscle cell oppose the acceptance of an adjacent atropine molecule? Does this signify that the three oxygen prosthetic groups repel the similar groupings of an adjacent molecule? This might prevent two atropine or eumydrin molecules from occupying adjacent positions on the cell surface except with extremely high dosage when laws of mass action might operate rather than a possible adsorption phenomenon. From a teleo-

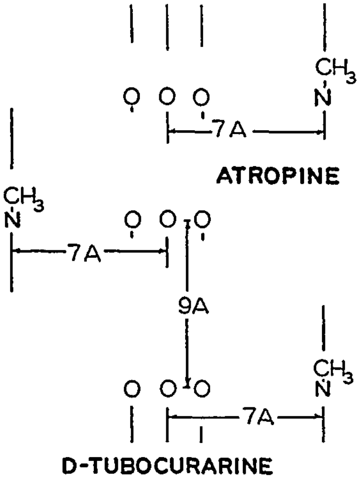


FIG. 2. DIAGRAMMATIC ARRANGEMENT WHICH DEPICTS THE PROSTHETIC GROUPS OF ATROPINE AND D-TUBOCURARINE

The distance between the nitrogens in d-tubocurarine is 13 to 15 A

logical viewpoint the omission of striated (voluntary) muscle from the blocking action of atropine-like compounds may indicate the possible existence (at one time at least) of an atropine-like controlling chemical in the body. For obvious reasons of "fright and flight" the voluntary muscles would of necessity be excepted.

While these studies point to the future synthesis of practical curare substitutes, none of the presently studied compounds have been sufficiently evaluated to suggest their use clinically as substitutes for curare. Due to the widespread action of these compounds on the neuromyal junctions the respiratory depression produced by large doses militates against curare-like compounds having a therapeutic index of more than two when the head drop dose is considered as the effective dose.

SUMMARY

Diatropines, whether synthesized by quaternization through the nitrogen atoms or by connecting the tropic acid hydroxyl groups by an aliphatic chain, have a markedly increased curare-like potency which approaches that of d-tubocurarine. Compounds with slight atropine-like activity become curare-like when two molecules are connected by an alkyl chain of suitable length. When the oxygen prosthetic groups are joined a chain of 9 A length should be used while a chain of 15 A length is probably optimal for joining the nitrogen atoms.

REFERENCES

1. Pfeiffer, C. C.: Science, **107**: 94, 1948.
2. Bovet, D., Courvoisier, S., Ducrot, R., and Horclois, R.: Compt. rend. Acad. Sci., **224**: 1733, 1947.
3. Loew, E. R., MacMillan, R., and Kaiser, M. E.: This Journal, **86**: 229, 1946.
4. Kimura, K. K., and Unna, K. R.: Fed. Proc., **7**: 233, 1948.
5. Litchfield, J. T., Jr., and Fertig, J. W.: Bull. Johns Hopkins Hosp., **69**: 276, 1941.
6. Barlow, R. B., and Ing, H. R.: Nature, **161**: 718, 1948.
7. Glock, G. E., Mogey, G. A., and Trevan, J. W.: Nature, **162**: 113, 1948.
8. Paton, W. D. M., and Zaimis, E. J.: Nature, **161**: 718, 1948.

COMPARATIVE PHARMACOLOGY OF THE OPTICAL ISOMERS OF ARTERENOL

F. P. LUDUENA, ESTELLE ANANENKO, O. H. SIEGMUND AND LLOYD C. MILLER

Sterling-Winthrop Research Institute, Rensselaer, New York

Received for publication October 19, 1948

Arterenol, 1-(3,4-dihydroxyphenyl)-2-aminoethanol or noradrenaline, and epinephrine were both synthesized in 1904 by Stolz and Flächer (1), and Dakin (2); a few years later Flächer succeeded in resolving epinephrine into its optical isomers (3). The study of the pharmacological actions of *l*- and *d*-epinephrine and related compounds disclosed the great biological importance of optical isomerism in the field of the sympathomimetic amines, and demonstrated that the *l*-isomers were responsible for most of the sympathomimetic activity of the racemic mixtures. Earlier studies with *d,l*-arterenol revealed that it has a stronger pressor action than *d,l*-epinephrine, and therefore *l*-arterenol was expected to be more active than the known sympathomimetic pressor agents. When one of the theories proposed for neurohumoral transmission of nerve impulses postulated the participation of *l*-arterenol, the importance of the latter was greatly increased. However, the studies on which this theory was based were carried out with the racemic compound since all attempts to separate the optical isomers were unsuccessful. When, in 1948, the resolution of arterenol was accomplished by Tullar (4) the long-sought *l*-isomer became available for pharmacological studies, and particularly for investigations of its role as a neurohumoral agent and of its potential value in therapeutics.

A preliminary report of the separation of the optical isomers of arterenol and a summary of a part of the pharmacologic results discussed here was recently published (5).

EXPERIMENTAL PART. *Blood Pressure.* *d,l*-Arterenol has been reported to be more active than *l*-epinephrine on the pressor response of the chloralosed cat previously sensitized with cocaine and ergotamine (6). A related sympathomimetic amine, Cobefrin (*d,l*-di-hydroxy-nor-ephedrine) has been found by Crismon and Tainter to be more active than both *d,l*-arterenol and *l*-epinephrine in stimulating the heart in the cat heart-lung preparation (7).

Most of the estimations of the relative potency of the sympathomimetic amines have been based on their cardio-vascular effects, and on this function arterenol and not epinephrine is the more active agent. Schultz (8) reported that *d,l*-arterenol exceeded *d,l*-epinephrine in pressor potency in a proportion of 1.5 to 1 on dogs under morphine-ether anesthesia. These results were confirmed by Barger and Dale (9) who found ratios of 1.25–1.5 to 1 and by Raymond-Hamet (10). On urethanized cats Tainter (11) calculated that *d,l*-arterenol was one-third more active than *d,l*-epinephrine. Recently West (12) confirmed this ratio using chloralosed cats.

METHOD. The pressor potencies of isomers of arterenol were determined on dogs anes-

thetized with phenobarbital, 150 mgm. per kgm., *intraperitoneally*. Carotid blood pressure was recorded by means of a mercury manometer. All injections were made into the femoral vein.

The USP XIII assay procedure for epinephrine was used, modified to the extent that both the standard and the "unknown" drug were given at two levels, the lower being about two-thirds of the higher, and no attempt was made to "match" the responses exactly. The four doses were given in an order determined by a Latin square as suggested by Noel (13) so that the responses to four sets of doses were obtained on each dog. The *l*-arterenol was in the form of the bitartrate monohydrate, the *d*-arterenol was the hydrochloride while the *l*-epinephrine was the USP Reference Standard Epinephrine, which is the base. *l*-Arterenol was assayed using *l*-epinephrine as the standard while *d*-arterenol was assayed against its *l*-isomer. The relative potencies and the weighted average were calculated by the methods

TABLE 1

Relative pressor potencies of l-arterenol, in terms of l-epinephrine, and d-arterenol, in terms of l-arterenol

Each value represents an assay on a separate dog

EXPT. NO.	*l* ARTERENOL* (*l* EPINEPHRINE = 100) PER CENT ± S.E.	EXPT. NO.	*d* ARTERENOL† (*l* ARTERENOL = 100) PER CENT ± S.E.
1	128 ± 3	7	3.0 ±0.2
2	186 ± 8	8	3.8 ±0.4
3	137 ± 7	9	3.9 ±0.3
4	161 ± 4	10	3.7 ±0.2
5	204 ± 15	11	3.7 ±0.3
6	223 ± 16		
Weighted average...............	158		3.64

* Injected as a solution of its bitartrate monohydrate salt and compared with an equimolecular quantity of USP Reference Standard Epinephrine.

† Injected as a solution of its hydrochloride salt and compared with an equimolecular quantity of *l*-arterenol bitartrate monohydrate.

described by Noel (13) and Miller, Bliss and Braun (14). Table 1 presents the results obtained on two groups of five dogs.

The data in the left-hand column of table 1 indicate that on an equi-molecular basis, *l*-arterenol is approximately 1.58 times as potent a pressor agent as *l*-epinephrine in dogs anesthetized with phenobarbital; on a weight basis, the ratio is 1.70. The standard errors of the individual assays are relatively low indicating a consistency in the respective responses of each dog to the two compounds. However, this consistency is not observed in the data as a whole since the variation from dog to dog is much greater than would be expected from the results on any one dog. The χ^2 test for homogeneity of the individual ratios (14) reveals that such discrepancies would occur through normal sampling much less frequently than once in a thousand times. The variations are a reflection of the qualitative difference between *l*-arterenol and *l*-epinephrine in their effects on the cardio-vascular system of the dog. Because of the heterogeneity, the weighted average of 158 per cent must be regarded with considerable reserva-

tion, the standard error of this average, calculated without regard to the heterogeneity, would be misleading and has been omitted

In the right-hand half of table 1 similar data on the relative potency of the two optical isomers of arterenol are summarized for five dogs anesthetized with sodium salts of Pentothal and barbital intravenously As these results indicate, *l*-arterenol is approximately 27 times more active than the *d* isomer and therefore is responsible for practically all of the pressor activity of the racemic mixture It will be noted that the variation between dogs in the pressor ratios of the two isomers of arterenol is much less than that observed between the two homologs, *l*-arterenol and *l* epinephrine

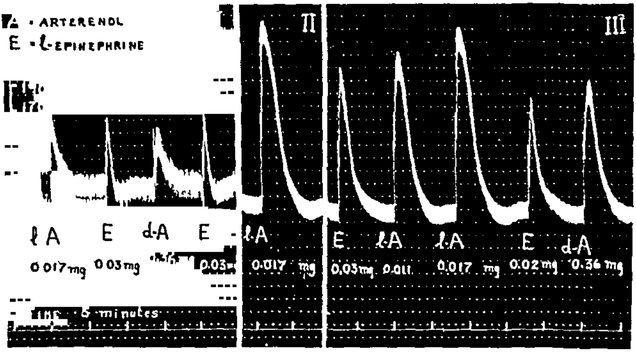


FIG 1 CAROTID BLOOD PRESSURE TRACINGS FOLLOWING THE INTRAVENOUS INJECTION OF *l*- AND *d* ARTERENOL (*d* A AND *l* A) AND *l* EPINEPHRINE (E)

Studies were made on an atropinized, 10 kgm dog anesthetized with 150 mgm /kgm, of phenobarbital intraperitoneally Between I and II a dose of 8 mgm /kgm of cocaine was injected subcutaneously

The pressor responses to equipressor doses of *l* epinephrine (1–2 microgm per kgm) and *l*- and *d*-arterenol differ somewhat in character In duration, the effect of *l*-epinephrine is the briefest, *l* arterenol intermediate and *d*-arterenol the longest, the mean ratios for the duration of equivalent pressor doses being in the ratios of 1:1.5:2.1 in dogs The duration of the pressor effect of *d*-epinephrine has also been found to be longer than that of the levo form (15, 16) Possibly this difference may be explained by a slower inactivation of *d*-arterenol, due either to the larger dosage or to a lesser affinity of the inactivating enzymatic system for the "unnatural" isomer

Confirming previous studies with racemic arterenol (Raymond-Hamet, 10, Tainter 11) it was found that the subcutaneous injection of 8 mgm of cocaine hydrochloride per kgm sensitized the pressor effects of *l*- and *d* arterenol Also in line with earlier observations on racemic arterenol and epinephrine (11, 17), the sensitization to *l* arterenol was greater than to *l*-epinephrine (fig 1) or *d*-arterenol

As an exception among the catecholethylamines, the pressor effect of racemic arterenol is not reversed by ergotoxine (Barger and Dale (9)), yohimbine (Raymond-Hamet (8)) or 933 F (N-piperidino-methyl-benzodioxane) (17). We have found that 2 mgm. per kgm. i.v. of the latter reversed the pressor effect of *l*-epinephrine but only reduced the effect of *l*- and *d*-arterenol.

Action on the heart. A few experiments were carried out on dogs anesthetized with Sodium Pentothal (15 mgm. per kgm.) and sodium barbital (250 mgm. per kgm.) intravenously in which the cardiac activity was recorded with a Jackson myocardiograph attached to the ventricles.

Cardiac acceleration and an increase in the amplitude of the contraction was observed with both *l*- and *d*-arterenol. Doses of *l*-arterenol of 0.3–1.0 microgm. per kgm. i.v., which produced increases in blood pressure of 20–50 mm. Hg, increased the cardiac amplitude from 40 to 300 per cent. The heart rate accelerated slightly with these doses in both normal and atropinized animals.

Equipressor doses of *l*-arterenol, *d*-arterenol and *l*-epinephrine had about the same quantitative effect on the amplitude of contraction (fig. 2). Crismon and Tainter have reported that *d,l*-arterenol is more active than *l*-epinephrine on the heart rate of the cat heart-lung preparation (7).

Perfused rabbit ear. The vasoconstrictor effect of *l*-arterenol was compared to that of *d*-arterenol and *l*-epinephrine on the perfused rabbit ear. The perfusion system was essentially that of Katz (19) as used by Moller to study the vasoconstrictor effect of cocaine (20). The ear artery is attached by a needle and rubber tubing to a vertical glass stand-pipe tube of 100 cm. height and 3 mm. internal diameter, with a funnel-like expansion blown at its upper end. From a Mariotte bottle, the perfusion fluid (Tyrode, at room temperature) falls dropwise into the upper end of the standpipe tube. The flow is regulated by a stopcock to 2.5 to 3.0 cc. per min.; at this rate the pressure is maintained at 25 to 40 cm. of water. As the rate of inflow is constant, any decrease in the caliber of the ear vessels results in a rise in the column of fluid, the magnitude of which is a measure of the vasoconstriction.

The absolute sensitivity and the discriminatory power of the preparation changes gradually during the experiment, increasing the error of quantitative determinations. Doses of 0.04–0.1 microgm. of *l*-epinephrine and 0.05–0.1 microgm. of *d,l*-arterenol injected into the tubing produced marked vasoconstriction. In the eight ears studied *l*-epinephrine was more active than *l*-arterenol with ratios varying from 1.5 to 2.5. On the other hand, *l*-arterenol was found to be from 12 to 18 times more active than the *d*-isomer by this test.

Prolongation of local anesthetic effect of procaine. The capacity of arterenol to extend the duration of local anesthesia was tested on rabbits by injection around the external canthus of the eye. Doses of 0.5 cc. of 0.5 per cent procaine alone and 0.5 per cent procaine with *l*-arterenol in dilutions varying from 1:100,000 up to 1:10,000 were injected and the anesthesia determined by the presence or absence of the corneal reflex.

The results in table 2 show that all the concentrations of arterenol used prolong the local anesthetic effect of procaine. However, a regular curve was not ob-

tained, probably due to the wide individual variations in response With both *l* epinephrine and *l* arterenol a plateau was reached between dilutions of 1 200 000 and 1 50 000 These data appear to confirm previous results obtained by Leser (22) with the same method in rabbits, who found no increase in the duration of anesthesia of procaine above a dilution of *l* epinephrine of

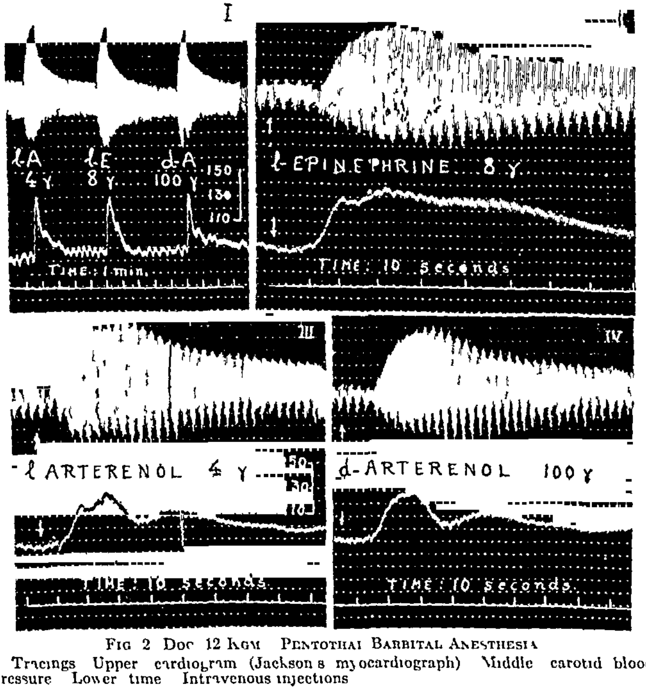


FIG 2 DOG 12 KGM PENTOTHAL BARBITAL ANESTHESIA

Tracings Upper cardiogram (Jackson's myocardiograph) Middle carotid blood pressure Lower time Intravenous injections

1 200 000 On the other hand by the intracutaneous wheal procedure in humans, Bieter (23) found that the optimum dilution of *l* epinephrine for increasing the local anesthetic effect of 0 125 per cent solution of procaine was 1 200,000 with stronger solutions resulting in a shorter duration of anesthesia Neither Leser nor Bieter tried *l* epinephrine 1 10,000, the strongest solution in our tests The greatest effect was obtained at this concentration with epinephrine and at 1 20,000 with arterenol At all the concentrations, the effect of epinephrine was more pronounced As measured by the duration of anesthesia, the vaso

constriction produced by *l*-epinephrine in the subcutaneous tissue and submucosa around the external canthus of the eye is more prolonged than that produced by equal doses of *l*-arterenol.

Intracutaneous wheal test in guinea pigs. The method of Bülbring and Wajda (24) consists in producing two intracutaneous wheals on the back of each animal by injecting the anesthetic solutions and determining the number of times there is no response to six pinpricks applied in sets to each wheal, every five minutes for 30 minutes. Six animals are used for each dose level. The negative responses obtained in the six readings for each wheal are summed and the mean for the six

TABLE 2

Comparison of l-epinephrine and l-arterenol in prolonging local anesthesia from 0.5% procaine in the external canthus of rabbits

VASOCONSTRICTOR NAME	DILUTION*	pD†	NUMBER OF RABBITS	DURATION IN MIN.	
				Ave.	Range
l-Arterenol....	1:1,000,000	6.0	12	36	25- 60
l-Arterenol	1:540,000	5.73	6	50	40- 60
l-Arterenol	1:400,000	5.6	6	72	65- 90
l-Arterenol	1:200,000	5.3	12	79	45-130
l-Arterenol	1:100,000	5.0	10	70	60- 85
l-Arterenol	1:54,000	4.73	5	79	70- 90
l-Arterenol	1:50,000	4.7	10	60	40- 90
l-Arterenol	1:27,000	4.43	6	82	60-115
l-Arterenol	1:10,000	4 0	11	127	85-155
l-Epinephrine	1:500,000	5.7	6	87	60-130
l-Epinephrine .	1:200,000	5.3	6	110	90-160
l-Epinephrine	1:100,000	5.0	10	124	65-190
l-Epinephrine	1:50,000	4.2	10	131	95-120
l-Epinephrine	1:25,000	4.4	10	110	70-140
l-Epinephrine	1:10,000	4 0	12	215+	135-300+
Procaine control	0 0		12	17	

* In terms of base.

† Log of reciprocal of the dilution ratio (21).

wheals is calculated. The maximum value of 36 was obtained with all four solutions, thereby indicating complete local anesthesia

In table 3 the values obtained after 30 minutes represent simply the arithmetic sums of six readings (one for each wheal). These results show the increase in duration of the anesthetic effect of procaine produced by both *l*-epinephrine and *l*-arterenol and at a dilution of 1:200,000 (in terms of the base) they were equally active.

Smooth muscle organs. Retractor penis in situ: The effect of *d,l*-arterenol on the dog's retractor penis has been studied by Barger and Dale (9), who found that it was considerably less active than *d,l*-epinephrine in producing contraction of this muscle. Other primary amines in their series were also found less active than the corresponding methylamines. Cocaine sensitizes the effect of epineph-

rine on the cat's retractor penis (25) and that of epinephrine and other catecholamines on the dog's retractor penis *in situ* (26)

Method Dogs anesthetized with Sodium Pentothal and sodium barbital were used In some animals, the sympathetic innervation of the muscle was suppressed by section of the lumbar sympathetic chains All the drugs were injected into the femoral vein and the resultant changes in carotid blood pressure recorded After longitudinal section of the skin covering the penis, the anterior part of the muscle was dissected, cut at the site of its anterior insertion and attached by a long thread to a heart lever for recording Equipressor doses of *l*- and *d*-arterenol produced approximately the same degree of contraction of the retractor penis Therefore, *l*-arterenol is about 27 times more active than the *d*-isomer on this muscle, however, in comparison with *l*-epinephrine, *l*-arterenol is much less active

TABLE 3

Intracutaneous wheal tests in guinea pigs with 0 1% procaine and epinephrine or arterenol

Method of Bulbring and Wajda (24)

VASOCONSTRICTOR AND DILUTION USED IN TERMS OF THE BASE	MEAN SUM FOR 30 MINUTES	INDIVIDUAL SCORES				
		60 min	90 min	120 min	150 min	175 min
l Epinephrine 1 200,000	36	34	30	25	20	
l-Epinephrine 1 100,000	36	36	36	36	33	33
l-Arterenol 1 400,000	36	34	26	24	18	17
l Arterenol 1 200,000	36	33	28	25	25	23
Procaine 0 1%	5 66 ±3 6					
Procaine 0 25%	16 0 ±4 2					
Procaine 0 5%	28 0 ±2 6					
Procaine 1 0%	31 6 ±4 1					

on the retractor penis than on blood pressure In three dogs, the dose of *l* arterenol required to induce the same degree of contraction was 4 to 5 times larger than that of *l* epinephrine This is illustrated in fig 3 in which the effect of equiactive doses of *l* epinephrine and *l*- and *d* arterenol can be compared

Isolated rabbit ileum The optical isomers of arterenol depress the activity of the isolated rabbit ileum Qualitatively they act like epinephrine In calculating the potency of *l*- and *d*-arterenol and *l*-epinephrine we have followed the method recently described by Miller, Becker and Tainter (21) for the determination of the activity of spasmolytic drugs, with the difference that we have used the unstimulated ileum Considered as an all-or-nothing response, a result was recorded as "positive" when the amplitude of the normal contractions was reduced by 75 per cent or more within two minutes Each dose level was tried on six to 12 strips from a minimum of six rabbits

The mean values of the ED_{50} in log dilutions (pD) were *d*-arterenol: 5 9 ± 0 07 (= 1:800,000), *l*-arterenol 7 70 ± 0 08 (1:50 millions) and *l* epinephrine 7 78 ± 0 12 (1.60 millions) Therefore, *l*-arterenol is about as active as *l*-epinephrine in producing inhibition In general agreement with the present

results Auman and Youmans (27) found *l*-epinephrine was 1.5 to 2.0 times as active as racemic arterenol and Emilsson (28) and West (12) observed a two-fold difference.

Isolated guinea pig ileum. Both optical isomers of arterenol relax the normal and the histamine-contracted isolated guinea pig ileum. The relative potencies of *d*- and *l*- arterenol and *l*-epinephrine in this respect were determined using at least six strips of the unstimulated ileum for each dose level by the method of Miller, Becker and Tainter.

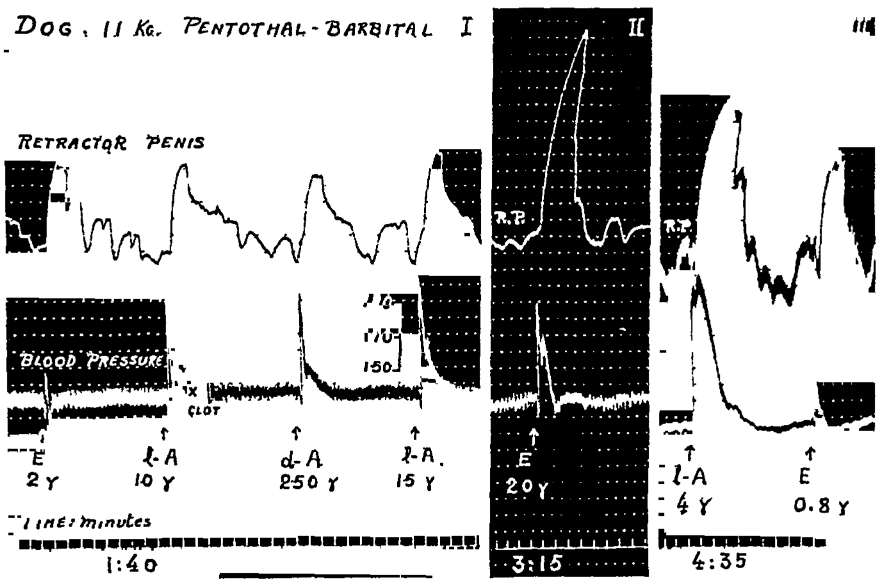


FIG. 3 DOG, 11 KGM. PENTOTHAL-BARBITAL ANESTHESIA

Atropinized Tracings. Upper. Retractor penis (abdominal sympathetic chains cut); middle. carotid blood pressure; Lower· time, 1 minute intervals. Injections into femoral vein E = *l*-epinephrine; *l*-A = *l*-arterenol, *d*-A = *d*-arterenol Total doses in mgm. Between II and III a dose of cocaine HCl was injected subcutaneously.

The guinea pig ileum was found to be more sensitive to these agents than the rabbit ileum. The pD producing positive results in 50 per cent of the strips was 6.58 ± .09 (1:3.8 millions) for *d*-arterenol, 8.02 ± .07 (1:105 millions) for *l*-arterenol and 8.16 ± .08 (1:144 millions) for *l*-epinephrine. Although the standard errors are relatively large, it appears that *l*-epinephrine is slightly more active than *l*-arterenol and that this is 25–30 times more active than the *d*-isomer, a ratio comparable to that obtained on the blood pressure of the barbitalized dog.

Isolated Rabbit Uterus (Non pregnant). The stimulating effects of *l*-arterenol and *l*-epinephrine were compared on the uterine horns of seven rabbits in dilutions of 1:10 million to 1:50 million. The two substances in the concentrations used produced the same degree of stimulation. The effect of *l*-arterenol appears to last longer

Guinea pig Uterus (Non pregnant). *l*-Epinephrine and *l*-arterenol were tested on the guinea pig uterus, either unstimulated or contracted by adding acetylcholine to give a 1:10 million dilution. The dilutions of the two amines varied from 1:2 million to 1:20 million. *l*-Arterenol was almost ineffective at 1:20 million. The erratic activity of the uteri made the estimation of the potency very difficult, it appears that *l*-epinephrine is from 2 to 10 times more active than *l*-arterenol in producing inhibition of the uterus.[1]

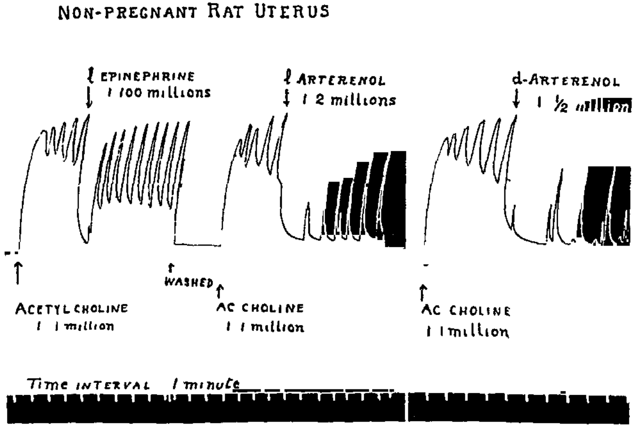


FIG 4 ISOLATED NON PREGNANT RAT UTERUS

Rat Uterus (Non pregnant). In the uterus stimulated by acetylcholine (1:1 million to 1:5 million, pD 6–6.7) *l*-arterenol was ineffective at 1:100 million. The threshold dilution seems to be around 1:10 million, a concentration three times this produced a degree of inhibition (decrease in tonus) comparable to that of 1:100 million of *l*-epinephrine. Therefore, *l* epinephrine is approximately 30 times more active than *l* arterenol in relaxing the rat uterus stimulated with acetylcholine. However, with equiactive doses, the effect of *l* arterenol was more prolonged. On the other hand, *l*-arterenol was about four times more active than *d* arterenol (fig. 4).

[1] In a new series of experiments, carried out in August, 1948, both *l* arterenol and *l* epinephrine were found to produce contraction instead of relaxation of non pregnant guinea pig uterus. Some investigators have obtained both contraction and relaxation of the pregnant as well as the non pregnant guinea pig uterus while others have observed either relaxation or contraction. (Literature reviewed by Gruber (44))

Effect on the Bronchioles. There is an extensive literature on the bronchiolar dilatation produced by the sympathomimetic amines. The effect of *d,l*-arterenol has been studied by Tainter *et al.* (29) on the perfused guinea pig lung. They found that it is only about $\frac{1}{7}$ as active on the average as *l*-epinephrine in relieving the spasms produced by histamine, pilocarpine or barium. On the lung *in situ* (30) with the Jackson method, *d,l*-arterenol is a good bronchodilator against the spasm produced by arecoline. On the perfused guinea pig lung, Siegmund, Granger and Lands (31) found that *l*-epinephrine was at least 10 times more active than *d,l*-arterenol against histamine bronchospasm. They also found *d,l*-arterenol very active on the spasm provoked by histamine inhalation. We have used both the perfused guinea pig lungs by the method of Sollmann and Von Oettingen (32) as modified by Tainter, Pedden and James (29) and the method devised by Schauman (33) with some modifications as described by Siegmund, Granger and Lands to induce bronchospasm by histamine inhalation.

TABLE 4

Bronchodilator action of d- and l-arterenol in comparison with l-epinephrine in histamine induced asthma in guinea pigs

DRUGS	DOSE* MGM./KGM. I.P.	NO. OF EXPERIMENTS	AVERAGE TIME†				PER CENT INCREASE		DOSAGE RATIO *l*-EPINEPHRINE = 1
			Control		Exper.				
			Onset	Duration	Onset	Duration	Onset	Duration	
l-epinephrine	0.01	7	0.60	1.04	1.09	1.46	73	35	1
l-epinephrine .	0.02	7	0.55	0.85	1.30	2.18	136	156	
l-arterenol	0.05	14	0.48	0.84	1.07	1.69	122	101	3
l-arterenol.	0.1	7	0.50	1.00	1.80	2.70	260	170	
d-arterenol	1.0	18	0.60	1.05	1.35	2.05	125	95	60

* All doses expressed in terms of the bases. Drugs were injected intraperitoneally.
† Time expressed in minutes.

On the perfused lung, *l*-arterenol in doses of 0.034 to 0.1 mgm. and *d*-arterenol in doses of 3 to 5 mgm. relaxed the bronchioles constricted by histamine (0.02–0.05 mgm.), all the drugs being injected into the perfusion fluid just entering the lung. The *l*-isomer was 50 to 60 times more active than the *d*-isomer, and about $\frac{1}{17}$ as active as *l*-epinephrine.

In the intact guinea pig, *l*- and *d*-arterenol showed antagonism to the spasm induced by inhalation of histamine diphosphate aerosol, as judged by the delays in the onset of initial symptoms and the appearance of asphyxial convulsions (duration). The results have been summarized in table 4.

By this method the ratios differ from those obtained with the perfused lung; *l*-arterenol is approximately 20 times more active than *d*-arterenol and about $\frac{1}{3}$ as active as *l*-epinephrine.

Central Nervous System Stimulation. The comparative activities of *l*-arterenol and *l*-epinephrine in stimulating the central nervous system have been investigated by the rat "jiggle-cage" technique of Tainter and co-workers (34). By

this method the movements of each rat are summated on an electric counter. *l*-Arterenol was administered subcutaneously as the *d*-bitartrate monohydrate salt and *l*-epinephrine as the base dissolved in weak hydrochloric acid solution, but all doses are expressed in terms of the free base for both compounds. A summary of the results is given in table 5.

From the results it may be seen that both *l*-arterenol and *l*-epinephrine produce moderate stimulation in the dose range used. The differences between the two drugs for individual intervals are scarcely beyond the limits of significance, in view of the relatively large standard errors. However, in the first three hours after injection all the hourly means obtained after *l*-epinephrine, with only one exception, are higher than the corresponding means (in time and dosage) for the *l*-arterenol rats. The total increase in revolutions over the control show greater differences inasmuch as the totals include the additional increases after

TABLE 5

Central nervous system stimulation produced in rats by l-epinephrine and l-arterenol as determined by the Jiggle Cage technique of Tainter, et al. (32)

DRUG	DOSE OF BASE MCM./KGM.	NO. RATS	AVERAGE NUMBER OF REVOLUTIONS PER HOUR ± S.E. AT TIMES SHOWN AFTER DOSING						TOTAL INCREASE IN REVOL. OVER CONTROL
			1 Hr.	2 Hr.	3 Hr.	4 Hr.	5 Hr.	6 Hr.	
Saline	(2 cc.)	22	2.3 ±0.5	1.4 ±0.2	1.6 ±0.3	1.1 ± 0.3	1.1 ±0.2	1.4 ±0.3	
l-Epinephrine	0.25	9	7.2 ±1.6	10.9 ±4.4	3.7 ±1.6	2.0 ±0.6	1.2 ±0.6	1.0 ±0.4	17.1
	0.75	12	10.6 ±2.4	8.8 ±2.3	6.0 ±1.3	3.1 ±0.9	1.6 ±0.6	1.7 ±0.5	22.9
	2.0	12	12.0 ±1.9	8.0 ±1.5	4.6 ±0.9	2.3 ±0.6	2.6 ±0.8	2.8 ±0.8	23.4
l-Arterenol	0.25	9	8.9 ±2.0	5.0 ±0.8	2.2 ±0.5	1.2 ±0.3	0.7 ±0.1	1.4 ±0.5	10.9
	0.75	12	9.6 ±2.0	5.3 ±1.6	1.8 ±0.6	0.6 ±0.2	0.5 ±0.2	0.7 ±0.2	11.4
	2.0	12*	7.7 ±1.3	4.0 ±1.0	1.4 ±0.7	1.2 ±0.3	0.9 ±0.3	0.9 ±0.06	7.9

* 1 animal of this group died.

the third hour following the injection. In conclusion it appears that *l*-epinephrine stimulates the central nervous system of the rat approximately twice as much as *l*-arterenol.

DISCUSSION. The results reported here on *l*-arterenol agree, in a general way, with prior knowledge of the pharmacological action of *d,l*-arterenol. As predicted, most of the activity of *d,l*-arterenol is attributable to the *l*-isomer. For arterenol, the average *d*:*l* pressor potency ratio is 1:27, which is intermediate between the ratios reported for the pairs of close analogues, namely: 1:12 to 1:15 (35, 36) and 1:18.5 (37) for *d*- and *l*-epinephrine and 1:30 (38) for *d*- and *l*-dihydroxynorephedrine. The relative potencies of the two optical isomers observed in various physiological structures or systems are summarized in table 6.

It is somewhat surprising to find that *l*-arterenol is as active as *l*-epinephrine on the small intestine of rabbits. It has been assumed that the primary amines in the catecholethyl- and propylamine series have appreciably less sympathomimetic inhibitor action than the corresponding methylamines.

Examination of table 6 shows that the ratios of *l*-arterenol potency to that of *l*-epinephrine bear no relation to whether an inhibitory or excitatory type of response is involved. *l*-Arterenol is more active on blood pressure and on the dog's heart. It is also more active on the pregnant uterus of cats according to reports in the literature (6, 12, 39). However, with other structures wherein the sympathetic is excitatory (dog's retractor penis and the cat's nictitating membrane (Bacq, 40)), *l*-arterenol is much less active than *l*-epinephrine. Arterenol is also less effective in raising the blood sugar in rabbits as demonstrated by Sahyun (41) with the racemic mixture and by McChesney and McAuliff with the *l*-isomer (42). In addition, *l*-arterenol is as potent as *l*-epinephrine on an inhibitory sympathetic structure (small intestine) while it is much less active on

TABLE 6

Relative potency of l-epinephrine and d- and l-arterenol

TEST OBJECT	*l*-ARTERENOL RATIO OF EQUIACTIVE DOSES OF *l*-ARTERENOL AND *l*-EPINEPHRINE	*d*-ARTERENOL RATIO OF EQUIACTIVE DOSES OF *d*- AND *l*-ARTERENOL	TYPE OF RESPONSE
	l-epinephrine = 1	*l-arterenol* = 1	
Dog, blood pressure	0.6	27	E*
Dog, heart in situ	0.6	27	E
Dog, retractor penis	4–5	27	E
Rabbit, ear perfusion (vasoconstriction)	1.5–2.5	12–18	E
Rabbit, isolated ileum	1	60	I†
Guinea pig, isolated ileum	1.5	27	E
Rabbit uterus, nonpregnant	1	—	E
Guinea Pig uterus, nonpregnant	2–10	—	I
Rat uterus, nonpregnant	30	4	I
Guinea Pig lungs			
a) perfusion	17	60	I
b) histamine asthma	3	20	I

* E—Excitation.
† I—Inhibition.

the two organs in which the sympathetic is excitatory (retractor penis and nictitating membrane). This raises the question whether *l*-arterenol can fulfill the theoretical requirements for an "excitatory" sympathin, as has been suggested by some investigators.

Originally "sympathetic receptive substance" or "myoneural junction" designated a specialized part of the sympathetic peripheral mechanism, through which the effect of the nerve impulses or of epinephrine was mediated, and which depended trophically on the effector cell, since it did not disappear after section and degeneration of the nerve fibers. It was actually a part of the effector cell, but different from the contractile substance upon which the so called "musculotropic" agents were supposed to act. In the newer concepts of neurohumoral transmission, the role played by the receptive substance changed but little; it continued to play the part of the trigger apparatus for the mechanical effect by

becoming the site of action of the ergone or ergones liberated at the periphery by the stimulation of the nerves As the effect of epinephrine and other closely related compounds on smooth muscle was augmentation or inhibition depending on the type of receptive substances involved (which in turn was revealed by the type of response to the stimulation of the sympathetic nerves) it was assumed that there are two receptive substances, excitatory and inhibitory, which are fundamentally different This assumption is supported by the fact that during pregnancy when the sympathetic supply of the uterus in the cat and other animals changes from inhibitory to excitatory there is a parallel change in the responses to epinephrine and closely related drugs

In the case of the organs in which the sympathetic is inhibitory there is no difficulty in accepting the hypothesis of the inhibitory receptive substance, although it should be considered as a working hypothesis until more is known about the mechanism of contraction of the smooth muscle cells However, the existence of sympathetic inhibitory innervation has been claimed to explain certain inhibitory effects produced by epinephrine and related compounds on the blood vessels, such as the fall of pressure elicited by small doses, the secondary hypotension which follows the normal pressor effect and the reversal obtained after sympatholytic agents Ergotoxine, 933 F and other sympatholytics should inhibit only the effect of sympathomimetic amines on the excitatory receptive substance, leaving the inhibitory component of the vascular effect unaltered, only those amines which act on both types of receptive substance should depress the blood pressure after sympatholytics Therefore, according to this theory, arterenol and other nor compounds have very little or no effect on the inhibitory sympathetic receptive substance The same theory was advanced to explain the fact that arterenol was more active than epinephrine on the blood pressure while it was less potent on the retractor penis (9) It was assumed that epinephrine was more active than arterenol in producing excitatory effects, as was shown on the retractor penis which has no inhibitory sympathetic supply, while on the blood pressure the pressor effect of epinephrine was reduced by its own inhibitory action, while the effect of arterenol, on the other hand, having a very weak or no inhibitory component, was fully effective This theory implies the existence of inhibitory sympathetic fibers in every vascular area in which epinephrine acts as a vasodilator after sympatholytics, a generalization for which there is no conclusive evidence, although inhibitory sympathetic fibers have been demonstrated for some vascular areas

Weight against this hypothesis is provided by the action of compounds such as N-isopropyl-arterenol (Isuprel) and other closely related amines which are predominantly or purely vasodilators in action Their action and the inhibitory effects of epinephrine are supposed to be similar in nature, that is, on inhibitory sympathetic receptive substances However, we have made the observation that Isuprel relaxes the atropinized dog's retractor penis *in situ* (fig 5) and *in vitro*, a muscle which does not have inhibitory sympathetic fibers (43) It also relaxes the atropinized vessels of the perfused ear of the rabbit, an animal in which ergotoxine does not reverse the pressor effect of epinephrine Apparently

he site of action of Isuprel on smooth muscle cells is not identified anatomically or physiologically with inhibitory sympathetic fibers and nerve endings. The site of the inhibitory action of epinephrine is presumably the same, considering he close chemical and pharmacological relationship of the two compounds.

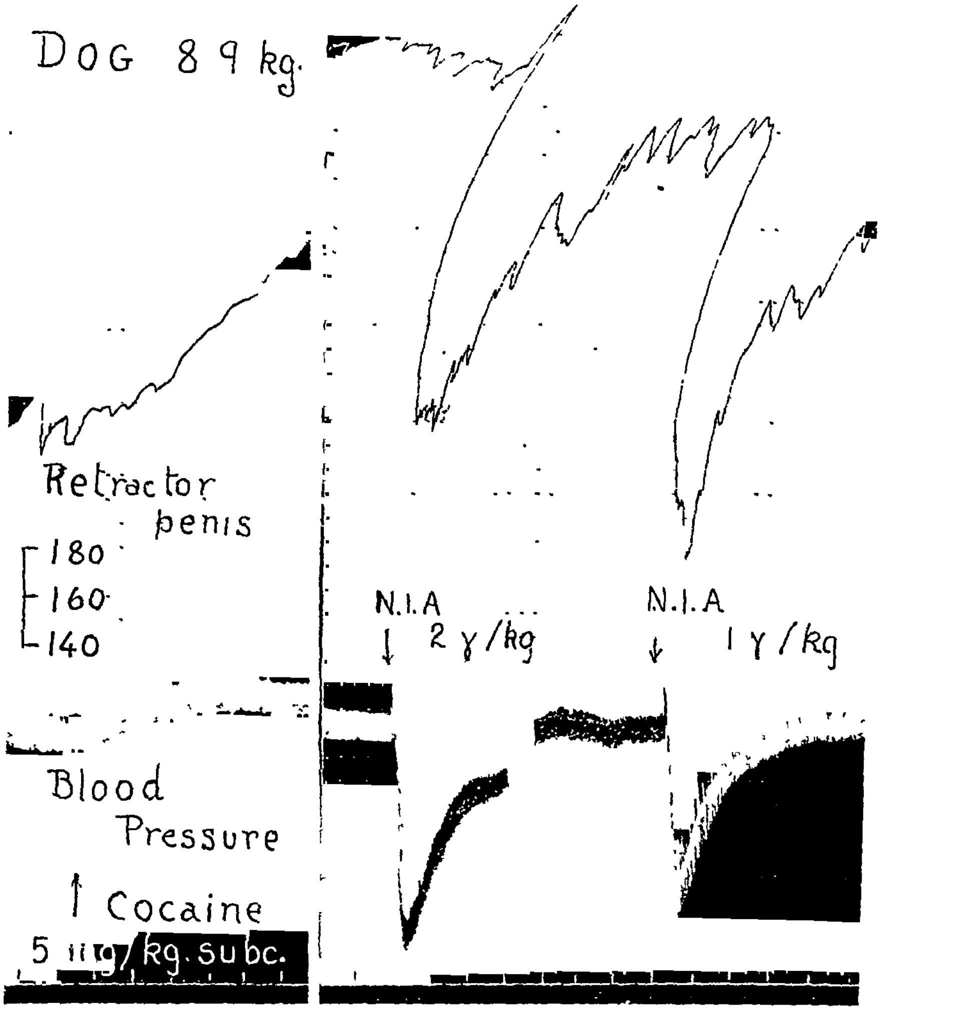


FIG 5. DOG, 8 9 KGM PENTOTHAL-BARBITAL ANESTHESIA

Tracings Upper retractor penis. Middle: carotid blood pressure Lower: time in minutes Intravenous injections. The tracings show the augmentor effect of cocaine and the inhibition produced by N-isopropyl-arterenol (Isuprel).

The fact that arterenol, epinephrine and Isuprel produce a combination of augmentation and inhibition of effector cells implies to us that in the chemical structure of the group of compounds known as sympathomimetic, the possibility exists of influencing in opposite directions the same biochemical mechanism which in the smooth muscle cell is responsible for or directly involved in the

mechanical responses. The affinity for the same cellular mechanism would depend on the structural features which they have in common while the type of amine group would be responsible for the direction and degree in which this mechanism is influenced.

CONCLUSIONS

1. The pharmacologic actions of *l*- and *d*-arterenol have been compared with *l*-epinephrine on the blood pressure, cardiac activity and the retractor penis of the barbitalized dog, on the isolated ileum of the rabbit and guinea pig, and the bronchioles of guinea pigs. The effects of *l*-arterenol were also compared to that of *l*-epinephrine on the uterus of rabbits and guinea pig, on the duration of the local anesthesia from procaine, and on the spontaneous random activity of the rat by the jiggle-cage technique.

2. On the barbitalized dog *l*-arterenol is 1.70 more active than *l*-epinephrine in raising blood pressure, while *d*-arterenol is about $\frac{1}{27}$th as active as the *l*-isomer. Equipressor doses of these drugs produce about the same degree of cardiac stimulation as judged by the increase in heart rate and amplitude.

3. On the dog's retractor penis *in situ*, equipressor doses of *l*- and *d*-arterenol *have the same stimulating effect. l-Epinephrine is 4 to 5 times more active than* *l*-arterenol.

4. Cocaine sensitizes the effect of *l*- and *d*-arterenol on the blood pressure and the retractor penis.

5. On the isolated rabbit ileum *l*-arterenol is approximately as active as *l*-epinephrine in producing inhibition: *d*-arterenol is about $\frac{1}{60}$th as active. On the isolated guinea pig ileum *l*-arterenol is somewhat less active than *l*-epinephrine in inducing relaxation while it is 20 times more active than *d*-arterenol.

6. The isolated rat uterus stimulated with acetylcholine is promptly relaxed by *l*- and *d*-arterenol. On this preparation, *l*-arterenol is about 4 times more active than the *d*-isomer and $\frac{1}{30}$th as active as *l*-epinephrine. In stimulating the isolated rabbit uterus *l*-arterenol and *l*-epinephrine are equally active, while the latter is 2 to 10 times more active than the former in inducing inhibition of the isolated nonpregnant guinea pig uterus.

7. In the perfused *guinea pig lung l-epinephrine is about 17 times more active* than *l*-arterenol which is 50 to 60 times more active than *d*-arterenol as a bronchodilator against histamine constriction. Against the bronchospasm induced by nebulized histamine in the intact guinea pig *l*-epinephrine is about 3 times more active than *l*-arterenol which is 20 times more active than *d*-arterenol.

8. *l*-Epinephrine is more potent than *l*-arterenol in increasing the duration of the local anesthetic effect of procaine.

9. Both *l*-epinephrine and *l*-arterenol moderately increase the spontaneous activity of the rat as judged by the jiggle cage method. The degree of stimulation is greater with *l*-epinephrine.

10. These results are not compatible, in any simple way, with certain current theories that identify *l*-arterenol as being the augmentor hormone of the sympathetic nervous system.

Acknowledgement. The authors are greatly indebted to Dr. M. L. Tainter,

who has had a long-standing interest in the isolation and evaluation of *l*-arterenol, for advice throughout this study.

The technical assistance of Mrs. Gwendolyn Jones, Miss Jacqueline Sherndal, Mrs. Ellen Miller, Mrs. Nettie Beglin and Mr. Calvin Zippin is gratefully acknowledged.

REFERENCES

1. Stolz, F., and Flächer, F.: D.R.P. 157,300 (Frdl., 7: 689); U. S. Patents No. 862,675; 930,703.
2. Dakin, H. D.: J. Physiol., 32: 34, 1905. Proc. Roy. Soc., Ser. B. 76: 491, 498, 1905.
3. Flächer, F.: Ztschr. f. physiol. Chem., 58: 189, 1908.
4. Tullar, B. F.: J. Am. Chem. Soc., 70: 2067, 1948.
5. Tainter, M. L., Tullar, B. F., and Luduena, F. P.: Science, 107: 39, 1948.
6. von Euler, U. S.: Acta Physiol. Scand., 12: 73, 1946.
7. Crismon, J. M., and Tainter, M. L.: This Journal, 64: 190, 1938.
8. Schultz, W. H.: Bull. 55, Hygienic Lab. U. S. Public Health, 1909.
9. Barger, G., and Dale, H. H.: J. Physiol., 41: 19, 1910.
10. Raymond-Hamet: Compt. rend. Acad. Sci., 191: 869, 1930.
11. Tainter, M. L.: Arch. internat. pharmacodyn. et de therap., 41: 365, 1931.
12. West, G. B.: J. Physiol., 106: 418, 1947.
13. Noel, R. H.: This Journal, 84: 278, 1945.
14. Miller, L. C., Bliss, C. I., and Braun, H. A.: J. Am. Pharm. A., 28: 644, 1939.
15. Frohlich: 1909, cit. by Sollmann, T.: Pharmacology, Sixth Edition, p. 422.
16. Tainter, M. L.: This Journal, 40: 43, 1930.
17. Melville, K. I.: This Journal, 59: 317, 1937.
18. Raymond-Hamet: Compt. rend. Soc. de Biol., 97: 618, 1927.
19. Katz, G.: Arch. internat. pharmacodyn. et de therap., 49: 239, 1934.
20. Moller, K.: Arch. internat. pharmacodyn. et de therap., 57: 51, 1937.
21. Miller, L. C., Becker, T. J., and Tainter, M. L.: This Journal, 92: 260, 1948.
22. Leser, A. J.: Anesthesiol., 1: 205, 1940.
23. Bieter, R. N.: Am. J. Surg., 34: 500, 1936.
24. Bülbring, E., and Wajda, I.: This Journal, 85: 78, 1945.
25. Oppenheimer, M. J.: Am. J. Physiol., 122: 745, 1938.
26. Luduena, F. P.: Rev. Soc. Arg. Biol., 14: 535, 1938.
27. Auman, K. W., and Youmans, W. B.: Proc. Soc. Exp. Biol. and Med., 42: 111, 1939.
28. Emilsson, B.: Acta Physiol. Scand., 3: 335, 1941.
29. Tainter, M. L., Pedden, J. R., and James, M.: This Journal, 51: 371, 1934.
30. Cameron, W. M., and Tainter, M. L.: This Journal, 57: 152, 1936.
31. Siegmund, O. H., Granger, H. R., and Lands, A. M.: This Journal, 90: 254, 1947.
32. Sollmann, T., and von Oettingen, W. F.: Proc. Soc. Exp. Biol. and Med., 25: 692, 1928.
33. Schaumann, O.: Arch. f. exp. Path. u. Pharmakol., 196: 109, 1940.
34. Schulte, J. N., Reif, E. C., Bacher, J. A., Jr., Lawrence, W. S., and Tainter, M. L.: This Journal, 71: 62, 1941.
35. Cushny, A. R.: J. Physiol., 38: 259, 1909.
36. Abderhalden, E., and Muller, F.: Ztschr. f. Physiol. Chem., 58: 185, 1908.
37. Swanson, E. E., Scott, C. C., Lee, H. M., and Chen, K. K.: This Journal, 78: 329, 1943.
38. Tiffeneau, M.: Congress de Physiol. Paris, 1920.
39. Greer, C. M., Pinkston, J. D., Baxter, J. H., Jr., and Brannon, E. S.: This Journal, 62: 189, 1938.
40. Bacq, Z. M.: Arch. internat. Physiol., 40: 297, 1935.
41. Sahyun, M.: Arch. internat. pharmacodyn. et de therap., 45: 285, 1933.
42. McChesney, E. W., and McAuliff, J. P.: unpublished results.
43. Luduena, F. P.: unpublished results.
44. Gruber, G. M.: Physiol. Rev., 13: 55, 1933.

EFFECTS OF TETRAETHYLAMMONIUM BROMIDE ON THE PARASYMPATHETIC NEUROEFFECTOR SYSTEM[1]

J V LUCO AND J MARCONI

Department of Pharmacology and Biochemistry, Catholic University of Chile

Received for publication October 21, 1948

Acheson and Pereira (1), in 1946, demonstrated that tetraethylammonium bromide blocks the synapse of sympathetic ganglia In the same year Acheson and Moe (2) interpreted some of the actions of this drug on the frequency of the heart beats as due to depression of the parasympathetic ganglion formations of the heart

It has been considered of interest to establish the possibility of blocking the ganglionic synapse of the parasympathetic system with the drug and also to study the effect of the drug on the transmission between the cholinergic postganglionic fiber and the effector, since adrenergic transmission is unhampered by the drug (1)

METHODS Cats anesthetized with an intraperitoneal injection of sodium pentobarbital urethane were used (sodium pentobarbital 0 03 grams to 1 cc of 25 per cent urethane 1 cc per kilogram of animal weight) A tracheal cannula was employed so that artificial respiration could be performed when necessary

The structures used were the superior cervical ganglion (sympathetic) and the ciliary ganglion (parasympathetic)

To stimulate parasympathetic preganglionic and postganglionic fibers, the method of Luco and Salvestrini (3) was employed The preganglionic and postganglionic sympathetic fibers were stimulated in the cervical region

The nerves were stimulated by short rectangular pulses of 0 0005 sec duration, the frequency of which was controlled by electronic valves Maximal stimulus was used in the majority of the experiments, and submaximal in the rest

The drug chosen—tetraethylammonium bromide (Kodak)—was administered intravenously (via the femoral vein) The action of the drug was recorded by photographing the variations in pupillary diameter at different times during stimulation

A total of 29 experiments was performed In nearly all the animals the adrenal glands were removed

RESULTS A) *Preganglionic parasympathetic fibers* The intravenous injection of tetraethylammonium bromide during stimulation of the third pair (preganglionic fibers of the ciliary ganglion) at frequencies that fluctuated between 30 and 60 per second and with maximal intensity produced a reduction or abolition of the pupillary reaction The effect can be observed 1 minute after the administration of the drug and lasts for a full 30 minutes

In nearly all the experiments the preganglionic sympathetic fibers were stimulated simultaneously on opposite sides, it was possible to observe a blocking of both pupillary reactions with doses fluctuating between 0 06 and 30 mgm per kilogram of body weight In one (fig 1), progressively larger doses were ad-

[1] Aided by the Ella Sachs Plotz Foundation, Boston, Massachusetts, and Fundacion Gildemeister, Santiago de Chile

who has had a long-standing interest in the isolation and evaluation of *l*-arterenol, for advice throughout this study.

The technical assistance of Mrs. Gwendolyn Jones, Miss Jacqueline Sherndal, Mrs. Ellen Miller, Mrs. Nettie Beglin and Mr. Calvin Zippin is gratefully acknowledged.

REFERENCES

1. Stolz, F., and Flächer, F.: D.R.P. 157,300 (Frdl., 7: 689); U. S. Patents No. 862,675; 930,703.
2. Dakin, H. D.: J. Physiol., 32: 34, 1905. Proc. Roy. Soc., Ser. B. 76: 491, 498, 1905.
3. Flächer, F.: Ztschr. f. physiol. Chem., 58: 189, 1908.
4. Tullar, B. F.: J. Am. Chem. Soc., 70: 2067, 1948.
5. Tainter, M. L., Tullar, B. F., and Luduena, F. P.: Science, 107: 39, 1948.
6. von Euler, U. S.: Acta Physiol. Scand., 12: 73, 1946.
7. Crismon, J. M., and Tainter, M. L.: This Journal, 64: 190, 1938.
8. Schultz, W. H.: Bull. 55, Hygienic Lab. U. S. Public Health, 1909.
9. Barger, G., and Dale, H. H.: J. Physiol., 41: 19, 1910.
10. Raymond-Hamet: Compt. rend. Acad. Sci., 191: 869, 1930.
11. Tainter, M. L.: Arch. internat. pharmacodyn. et de therap., 41: 365, 1931.
12. West, G. B.: J. Physiol., 106: 418, 1947.
13. Noel, R. H.: This Journal, 84: 278, 1945.
14. Miller, L. C., Bliss, C. I., and Braun, H. A.: J. Am. Pharm. A., 28: 644, 1939.
15. Frohlich: 1909, cit. by Sollmann, T.: Pharmacology, Sixth Edition, p. 422.
16. Tainter, M. L.: This Journal, 40: 43, 1930.
17. Melville, K. I.: This Journal, 59: 317, 1937.
18. Raymond-Hamet: Compt. rend. Soc. de Biol., 97: 618, 1927.
19. Katz, G.: Arch. internat. pharmacodyn. et de therap., 49: 239, 1934.
20. Moller, K.: Arch. internat. pharmacodyn. et de therap., 57: 51, 1937.
21. Miller, L. C., Becker, T. J., and Tainter, M. L.: This Journal, 92: 260, 1948.
22. Leser, A. J.: Anesthesiol., 1: 205, 1940.
23. Bieter, R. N.: Am. J. Surg., 34: 500, 1936.
24. Bülbring, E., and Wajda, I.: This Journal, 85: 78, 1945.
25. Oppenheimer, M. J.: Am. J. Physiol., 122: 745, 1938.
26. Luduena, F. P.: Rev. Soc. Arg. Biol., 14: 535, 1938.
27. Auman, K. W., and Youmans, W. B.: Proc. Soc. Exp. Biol. and Med., 42: 111, 1939.
28. Emilsson, B.: Acta Physiol. Scand., 3: 335, 1941.
29. Tainter, M. L., Pedden, J. R., and James, M.: This Journal, 51: 371, 1934.
30. Cameron, W. M., and Tainter, M. L.: This Journal, 57: 152, 1936.
31. Siegmund, O. H., Granger, H. R., and Lands, A. M.: This Journal, 90: 254, 1947.
32. Sollmann, T., and von Oettingen, W. F.: Proc. Soc. Exp. Biol. and Med., 25: 692, 1928.
33. Schaumann, O.: Arch. f. exp. Path. u. Pharmakol., 196: 109, 1940.
34. Schulte, J. N., Reif, E. C., Bacher, J. A., Jr., Lawrence, W. S., and Tainter, M. L.: This Journal, 71: 62, 1941.
35. Cushny, A. R.: J. Physiol., 38: 259, 1909.
36. Abderhalden, E., and Muller, F.: Ztschr. f. Physiol. Chem., 58: 185, 1908.
37. Swanson, E. E., Scott, C. C., Lee, H. M., and Chen, K. K.: This Journal, 78: 329, 1943.
38. Tiffeneau, M.: Congress de Physiol. Paris, 1920.
39. Greer, C. M., Pinkston, J. D., Baxter, J. H., Jr., and Brannon, E. S.: This Journal, 62: 189, 1938.
40. Bacq, Z. M.: Arch. internat. Physiol., 40: 297, 1935.
41. Sahyun, M.: Arch. internat. pharmacodyn. et de therap., 45: 285, 1933.
42. McChesney, E. W., and McAuliff, J. P.: unpublished results.
43. Luduena, F. P.: unpublished results.
44. Gruber, G. M.: Physiol. Rev., 13: 55, 1933.

graph) and after stimulation (bottom one) are due to the absence of the light reflex by the blocking effect of the drug on the ciliary ganglion An intense effect on the sympathetic ganglion appears after the first injection at B and the complete blocking effect after the second one at C, as proved by the fact that a double dose administered at D does not modify the pupillary diameter and by the fact that it is equal to the diameter after stimulation In the parasympathetic ganglion the effect is observed only after the second injection, and it is not yet complete after the third one, i e , the pupillary diameter is smaller than the one before or after stimulation

In other words, the effect on the parasympathetic ganglion was obtained in this experiment with doses about four times larger than the one required to block the sympathetic ganglion

In other experiments the ciliary ganglion was acutely removed on the same side as the sympathetic stimulation, and the blocking effects were of the same magnitude as the experiment of fig 1

FIG 2 DEPRESSOR AND POTENTIATION EFFECTS WITH SMALL AND LARGE DOSES DURING STIMULATION OF THE POSTGANGLIONIC PARASYMPATHETIC FIBERS WITH MAXIMAL STIMULI

A and E show the beginning and ending of stimulation At B, C and D, tetraethylammonium bromide 3 mgm , 8 mgm and 160 mgm per kilogram of body weight, respectively

B) *Postganglionic parasympathetic fibers* During stimulation of the postganglionic parasympathetic (cholinergic) fibers at a frequency of 25 to 50 per second, various effects can be observed according to the dose used· With 10 to 100 mgm per kilogram of body weight, block of the response of the effector is observed similar to the autonomic ganglion effect

With doses between 200 and 300 mgm per kilogram of body weight, it is often observed that an increase in the pupillary contraction takes place After this potentiation of the response, which lasts nearly 1 minute, a depression and blocking is observed, as shown by a prolonged pupillary dilatation

The depression with small doses and potentiation with large ones has been observed with both maximal and submaximal stimuli (figs 2 and 3) The depressor effect can be diminished if the intensity of the stimulus is increased, regardless of whether maximal or submaximal stimulation is employed, but the increase was never greater than that obtained with a maximal stimulus

In 2 experiments it was observed, as reported by Acheson and Pereira (1), that the response of smooth muscle to acetylcholine (in this case the pupillary sphincter) is not altered by the previous administration of the drug (fig 4)

C) *Direct effect on the pupil.* It was considered interesting to know the effect produced by direct action of the drug on the pupil. Four cats were used. In one the sympathetic and ciliary ganglia of both sides were acutely removed, and in the other three the superior cervical ganglion of one side was excised 5 to 7 days previously and the rest of the ganglia acutely removed. Under these conditions the drug produces a slight increase of the pupillary diameter (fig. 5)—a

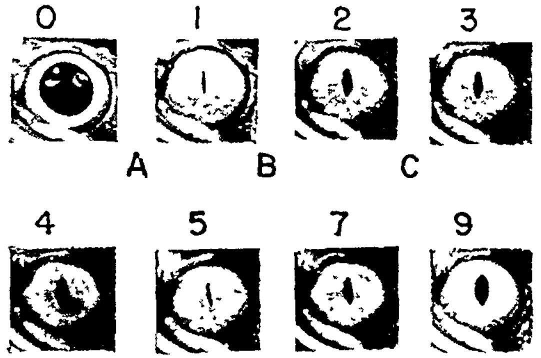


FIG. 3. POTENTIATION WITH LARGE DOSES DURING SUBMAXIMAL STIMULATION OF THE POSTGANGLIONIC PARASYMPATHETIC FIBERS

At A, stimulation begins, with maximal stimuli. At B, intensity is reduced (submaximal). At C, injection of tetraethylammonium bromide: 160 mgm. per kilogram of body weight.

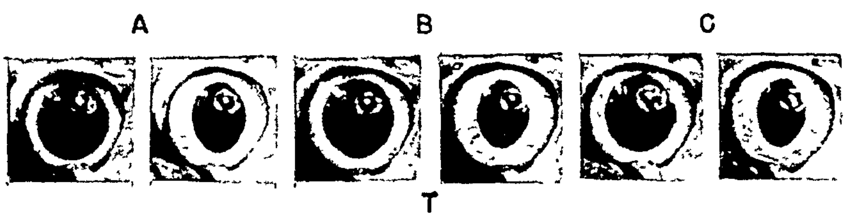


FIG. 4. EFFECT OF THE DRUG ON ACETYLCHOLINIC RESPONSE OF THE PUPIL

Chronic sympathetic postganglionic denervation (6 days) and acute decentralization (removal of ciliary ganglion). At A, B and C, injections of 50 micrograms of acetylcholine. T shows intravenous injection of 16 mgm. per kilogram of body weight of tetraethylammonium bromide, immediately before B.

reaction that is, of course, more intense when the initial tension of the pupil is greater.

In the different experiments, it was never observed that the drug produced pupillary contraction *per se*, even with doses as large as 300 mgm. per kilogram. From this, one may infer that the drug has no muscarinic effect, confirming previous reports, such as those cited by Acheson and Moe (2).

DISCUSSION. Luco and Mesa (4) demonstrated that curare produces a blocking of the synapse in which acetylcholine is mediator. Tetraethylammonium

bromide has a similar action on the neuroeffectors studied, thus Acheson and Pereira (1) observed a depressor effect on the sympathetic ganglion, and this paper studies its action on parasympathetic ganglia and on the postganglionic cholinergic effector system The observation of Acheson and Pereira that, like curare, it does not block the postganglionic adrenergic effector system is confirmed

The dilatation of the pupil observed with the administration of the drug, while stimulating the ciliary fibers, is not due to a direct effect of tetraethylammonium bromide on the pupillary sphincter, since during postganglionic sympathetic stimulation there is no effect of the drug on the dilator of the pupil On the other hand, the magnitude of direct effect of the drug on the pupil dilatation is small

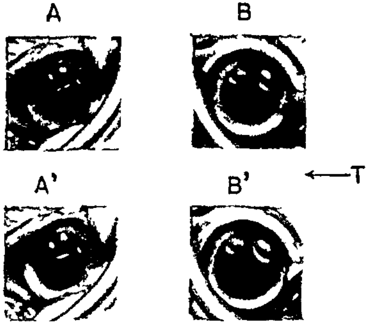


Fig 5 Direct Effect of the Drug on the Pupil

A and A′ (right side) show the acutely decentralized pupil B and B′ (left side), the acutely decentralized and chronically denervated pupil (removal of the superior cervical ganglion) At T, injection of tetraethylammonium bromide 25 mgm per kilogram of body weight

whereas the depression during postganglionic stimulation may be complete These considerations therefore exclude the possibility that the depressor effect is due principally to a change in the smooth-muscle contractile system

It does not at present seem likely that the drug has an effect on the post ganglionic axon, since no depression of the postganglionic sympathetic fibers is observed

It is not possible to accept an increase in the threshold to acetylcholine, which would easily explain the depressor effect, since both in the experiments of Acheson and Pereira (1) and in these, the threshold of the nictitating membrane and the pupil to acetylcholine remained unaltered

The possibility of a change in the liberation of the mediator would explain the results obtained, but this interpretation has not been submitted to experimental proof

The transitory, but constant, potentiation produced by the drug in high doses during stimulation is not due to direct action on the muscle, since such a reaction has never been observed, as pointed out in Section C above. This increase in the contraction could also be explained as due to an alteration in the liberation of the mediator.

SUMMARY

The effect of tetraethylammonium bromide on the parasympathetic ganglion and the postganglionic cholinergic effector system was studied on cats anesthetized with sodium pentobarbital.

During stimulation of the preganglionic fibers of the ciliary ganglion, a blocking of the transmission of the impulse through the ganglion was observed.

The stimulation of the postganglionic fibers revealed a blocking of the pupillary response with intravenous doses of 10 to 100 mgm. per kilogram of body weight, and a potentiation of the response with doses from two to three times larger.

REFERENCES

(1) Acheson, G. A., and Pereira, S. A.: This Journal, 87: 273, 1946.
(2) Acheson, G. A., and Moe, G. K.: This Journal, 87: 220, 1946.
(3) Luco, J. V., and Salvestrini, H.: J. Neurophysiol., 5: 27, 1942.
(4) Luco, J. V., and Mesa, J.: Ciencia (Méjico), 2: 298, 1941.

THE ADRENERGIC BLOCKING EFFECT OF CERTAIN β CHLOROETHYL AMINES[1]

CARLTON C. HUNT[2]

WITH THE TECHNICAL ASSISTANCE OF DORIS ROSENTHAL

Department of Pharmacology, Cornell University Medical College, and the Pharmacology Section, Sloan Kettering Institute for Cancer Research, New York

Received for publication October 21, 1948

Many compounds, both natural and synthetic, have been found to possess adrenergic blocking activity The clinical application of such agents has been limited by their toxicity and brief duration of action In 1946 Nickerson and Goodman (1) reported that dibenzyl β chloroethyl amine (dibenamine) was capable of blocking certain adrenergic responses for a period up to several days after a single dose Subsequently Loew, Kaiser, and Anderson (2) described the similar effects of other β halogenated ethyl amines, namely· benzhydrylethyl β-chloroethyl amine, β-biphenoxyethyl β chloroethyl amine, and α napthylmethyl ethyl β chloroethyl amine

This group of compounds is presumed to undergo intramolecular cyclization under physiological conditions with the formation of a highly reactive imine ring It is to this transformed state of the dibenamine molecule that the adrenergic blocking effect has been ascribed (3) This effect can be prevented by the prior administration of thiosulfate, an action which is attributed to the ability of this ion to combine with the imine form of such compounds with the production of an inactive ethyl thiosulfate derivative

The present study is concerned with the relationship between chemical structure and adrenergic blocking activity of a number of β chloroethyl amines and with certain aspects of the mechanism of action of such compounds

METHODS *Mouse Protection Test* The ability of adrenergic blocking agents to protect mice against the lethal effects of epinephrine has been utilized as a test for such activity (2) In the present study albino mice were injected subcutaneously with the compound to be tested and the LD_{80-100} of epinephrine[3] (20 mgm /kgm) was subsequently injected intra peritoneally Groups of 10 mice were used in each experiment and frequent control deter minations of epinephrine toxicity were made All injections were made in a volume corre sponding to 0 1 cc /10 grams body weight, except in the case of epinephrine which was given in a volume of 0 2 cc /10 gram The compounds tested were hydrochloride salts dissolved in normal saline (sometimes slightly acidified) or in propylene glycol diluted with normal saline The number of mice per cage and the type of cage were kept constant

---

[1] The work described in this paper was done in part under contract between the Medical Division, Chemical Corps, U S Army, and Cornell University Medical College Under terms of the contract, the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors The investigation was also supported by research grants from the U S Public Health Service, National Institute of Health, Division of Research Grants and Fellowships, and from the American Cancer Society

[2] Present address Dept of Medicine, Johns Hopkins Hospital, Baltimore

[3] Commercially prepared 1 1,000 solution of the hydrochloride salt

Blood pressure. Adult cats were anesthetized with 0.5 cc./kgm. of "Dial" solution (Ciba) administered intraperitoneally. The carotid artery was cannulated and the blood pressure recorded with a mercury or Hürthle manometer. All injections were made into the saphenous vein.

Nictitating membrane. The response of the nictitating membrane was recorded by attachment to an isotonic lever. Silver electrodes were placed on the cervical sympathetic trunk and stimulation effected by an inductorium.

Cross-circulation experiments. In 4 experiments cross-circulation was established in a pair of cats through glass cannulae which connected the cardiac end of the common carotid artery of each animal with the cardiac end of the external jugular vein of the other. Heparin was used as the anti-coagulant. Dibenamine was injected intravenously into 1 (donor) cat with the cross-circulation interrupted. After an interval, cross-circulation was established. Subsequent to this, with the cross-circulation again interrupted, the blood pressure response of the second (recipient) cat to epinephrine was recorded through a cannula in the femoral artery.

Isolated organs. A glass organ bath with a volume of 40 cc. was maintained at 38° C. A 4–5 cm. strip of freshly removed rabbit intestine or non-pregnant rabbit uterus was connected in the bath to a heart lever. A Krebs-Ringer bicarbonate medium was used containing 0.1 per cent glucose. A mixture of 95 per cent O_2 and 5 per cent CO_2 was bubbled continuously through the bath.

Papillary muscle. The technique of Cattell and Gold (4) was used. A Ringer-bicarbonate solution was gassed continuously with 95 per cent O_2 and 5 per cent CO_2. The muscle was stimulated at a rate of 1/sec. and responses were recorded photographically.

RESULTS. *Relationship of structure to adrenergic blocking activity.* A series of β-chloroethyl amines was studied for a protective effect against lethal doses (LD_{80-100}) of epinephrine in mice. Table 1 shows the results of 20 compounds so tested. After an initial dose of the β-chloroethyl amine (usually 20 mgm./kgm.) progressively smaller doses were tested until $\frac{4}{10}$ or more of a group of mice died from epinephrine. A few compounds reported previously (2, 3) are included for comparison. Those agents which failed to afford protection when epinephrine was given 30 minutes later were retested, allowing an interval of 2 hours between administration of the drug and of epinephrine.

Nickerson, Nomaguchi, and Goodman (3) reported that p-propyl or chlor substitution on the benzyl groups of dibenamine abolished activity, and that at least one benzyl group was essential for activity and could not be replaced by a phenyl, phenylethyl, or aliphatic group without complete inactivation. However, of the *bis* β-chloroethyl amines reported here, the benzyl, β-phenylethyl, and γ-phenylpropyl derivatives were active. Since benzhydrylethyl and β-biphenoxyethyl β-chloroethyl amines are also active, it appears that more than one C atom may be interposed between the N atom and the aromatic group in active compounds.

Of the agents tested for a protective effect in mice, α-napthylmethyl ethyl β-chloroethyl amine appeared the most active. Any absolute comparison of the potency of these compounds in this regard would require the determination of the time taken for the development of maximal effect for each compound. This is evident in the case of γ-phenylpropyl *bis* β-chloroethyl amine which offered no protection when lethal doses of epinephrine were given 30 minutes later, but did protect against epinephrine administered 2 hours later. Thus any compari-

son of potency of adrenergic blocking agents reported herein is valid only for the particular time interval studied.

The finding that β-chloro β-phenylethyl amines may possess adrenergic blocking activity is of special interest. Dimethyl β-chloro β-phenylethyl amine was an active protective agent, the first demonstration that the aromatic group may be on the chloralkyl group in active compounds. This compound also showed striking cholinergic effects following intravenous administration in cats, causing: an acute flaccid paralysis, skeletal muscle fasciculations, a fall in blood pressure which was blocked by atropine, a depression of the responses of the intact cat gastrocnemius preparation to maximal motor nerve stimuli, and contraction of the nictitating membrane. This compound appears to undergo rapid cyclization at neutral pH (0.02 *M* solutions in 0.16 *M* $NaHCO_3$ attained maximal Cl^- evolution in 2 minutes) and its cholinergic effects may be ascribed to the resultant quaternary onium cation. These effects closely resemble those following the administration of methyl β-chloroethyl ethylenimonium ion to cats (5).

Evidence of adrenergic block in cats was manifest by reversal of blood pressure responses to injected epinephrine and by a reduction in the contractile responses of the nictitating membrane to cervical sympathetic stimulation. The latter was the more resistant to block. All of the compounds tested which had shown a protective effect in mice, caused some degree of adrenergic block in cats. There was little correlation of the potency on the 2 test objects, although larger doses were always required to induce adrenergic block in cats (table 1). In some cases reversal of the blood pressure responses to epinephrine was incomplete and the use of higher doses of the agent was prevented by its toxicity (dimethyl β-chloro β-phenylethyl amine). In general, all the adrenergic blocking agents tested blocked vasopressor responses to injected nor-epinephrine and epinephrine and had little effect on vasodepressor responses to epinephrine and isopropyl nor-epinephrine. In an occasional cat nor-epinephrine caused a slight fall in blood pressure after dibenamine.

Response to injected epinephrine was tested up to 30 minutes after a single dose of an agent. This would cause a low estimate of the potency of compounds which attain maximal effect more slowly than dibenamine.

Isolated tissues. Rabbit duodenum. As reported by Nickerson and Goodman (6), dibenamine had no effect on the reaction of this preparation to epinephrine. However in concentrations of 1:10,000 benzyl *bis* β-chloroethyl amine and α-napthylmethyl *bis* β-chloroethyl amine prevented the inhibition of the spontaneous contractions caused by epinephrine. The significance of this *in vitro* effect is uncertain.

Rabbit uterus. Non-pregnant rabbit uterus showed contractile responses to epinephrine which were completely blocked by dibenamine (1:100,000 and less). This effect was resistant to repeated washing.

Papillary muscle. While Nickerson and Goodman (6) found that dibenamine had no effect on the positive inotropic action of epinephrine as judged by changes in pulse pressure, it was of interest to test this directly using the papillary muscle

technique. Dibenamine had no effect on the excitatory response to epinephrine, *nor-epinephrine*, or *isopropyl* nor-epinephrine (table 2). Benzyl *bis* β-chloro-

TABLE 1

Mortality of LD_{80-100} of epinephrine in mice after certain 2-chloroethyl amines

R	R′	INT.*	DOSE (MGM./KGM.) 20	10	5	2.5	1.0	0.5	0.25	0.01	ADRENERGIC BLOCK—CAT†
Mono-2-chloroethyl amines: $R(R')N{-}CH_2CH_2Cl$											
benzyl	benzyl[a, b]	30	0/30	0/10	5/10						10
o-methylbenzyl	o-methylbenzyl[a, b]	30	0/10	5/10							15 (inc.)
		120	10/10								
m-methylbenzyl	m-methylbenzyl[a, b]	30	0/10	0/10	4/10						
o-chlorbenzyl	o-chlorbenzyl[a, b]	30	10/10								
		120	9/10								
p-isopropylbenzyl	p-isopropylbenzyl[a, b]	30	9/10								
α-naphthylmethyl	ethyl[c]	30	0/20	0/10	0/10	0/20	0/10	0/10	3/20	8/10	5–6
methyl	methyl[c]	30	10/10								20 (inc.)
		120	10/10								
phenyl	ethyl[d]	30	7/10								
		120	9/10								
2-chloro, 2-phenylethyl amines: $R(R')N{-}CH_2CHCl{-}C_6H_5$											
methyl	methyl[a]	*30*	*1/10*	*0/10*	*0/20*	*0/10*	*0/10*	*5/10*			2 (inc)
benzyl	benzyl[a, b]	30	10/10								
Bis 2-chloroethyl amines: $R{-}N{=}(CH_2CH_2Cl)_2$											
phenyl[d]		*30*	*10/10*								
		120	10/10								
benzyl[b]		30	3/10	2/10	0/10	0/10	0/10	0/10	4/10	8/10	8
β-phenylethyl[d]		30	0/10	3/10							10
γ-phenylpropyl[d]		30	10/10								
		120		1/10	0/10	1/10	0/10	1/10	5/10		
o-chlorobenzyl[d]		30	4/10	4/10	7/10						
p-chlorobenzyl[a, b]		30	7/10								
		120	9/10								
α-naphthylmethyl[c]		30	1/10	0/10	0/10	0/10	0/10	0/10	4/10		
p-nitrobenzyl[a, b, d]		30	10/10								
		120	9/10								
methyl[b, d]		30	7/10								

* Interval between administration of agent and injection of epinephrine.
† Approximate dose (mgm./kgm.) causing epinephrine reversal.
The author gratefully acknowledges samples of compounds from the following sources:
[a] The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, Ind.
[b] Chemical Corps, U. S. Army.
[c] Parke, Davis and Company, Detroit, Michigan.
[d] Merck & Company, Inc., Rahway, N. J.

ethyl amine was also without effect. These agents alone did not influence the contraction of the papillary muscle.

Duration of the adrenergic blockade. Cross-circulation. Experiments were performed in cats to test the possibility that circulating dibenamine might per-

sist for some time after intravenous administration One of a pair of cats was given a dose of dibenamine intravenously which corresponded to 20 mgm /kgm

TABLE 2

Response of the isolated papillary muscle to epinephrine after adrenergic blocking agents

MUSCLE	DRUG	CONCENTRATION	CHANGE IN RESPONSE (MM)
#1	Epinephrine	1 1,000,000	10 → 24
	Benzyl bis β chloroethyl amine	1 500,000	
	+ Epinephrine	1 1,000,000	10 → 28
	Benzyl bis β chloroethyl amine	1 250,000	
	+ Epinephrine	1 1,000,000	12 → 32
#2	Epinephrine	1 1,000,000	7 → 22
	α napthylmethyl ethyl β chloroethyl amine	1 500,000	
	+ Epinephrine	1 1,000,000	5 → 23
#3	Epinephrine	1 1,000,000	17 → 22
	Dibenamine	1 500,000	
	+ Epinephrine	1 1,000 000	8 → 22
	Dibenamine	1 50,000	
	+ Epinephrine	1 1,000,000	4 → 17

TABLE 3

Cross circulation experiments in cats

EXPERIMENT	INTERVAL AFTER DIBENAMINE BEFORE MIXING	DURATION OF CROSS CIRCULATION	BLOOD PRESSURE RESPONSE OF RECIPIENT CAT (MM.Hg) TO EPINEPHRINE (MICROGM/KGM)		
			10	30	100
	min	*min*			
A	—	0	+66 (control)		
	15	30	+26	+26	
B	—	0	+66		
	40	30	−20	−32 → +31	−26
C	—	0	+106		
	40	30	+20	+53	
D	—	0	+100		
	270	30	+59		

of the weight of both cats After an interval the circulations were allowed to mix, again interrupted, and the recipient cat tested for evidence of adrenergic block by the blood pressure response to injected epinephrine The results are shown in table 3 Definite evidence of adrenergic block in the recipient cat was

obtained 15 and 40 minutes after the administration of dibenamine to the donor, and in one cat an effect was still present after 4.5 hours. Mixing of a dye which is confined to the vascular space is complete in this preparation after 5 minutes of cross circulation (7). Cross circulation alone had no effect on the blood pressure at about the same level. These findings indicate that circulating dibenamine may persist for several hours after a single dose.

Protection experiments in mice. Additional evidence for the persistence of dibenamine is provided by experiments which utilized the ability of thiosulfate ion, when administered prior to dibenamine, to prevent the development of adrenergic block. Thiosulfate ion is distributed to the extracellular space and is rapidly cleared by the kidney (8). Cats given sodium thiosulfate 0.5

TABLE 4

*Mortality of LD_{80-100} of epinephrine in mice receiving sodium thiosulfate followed by adrenergic blocking agents**

INTERVAL BETWEEN AGENT AND EPINEPHRINE (MIN.)	DIBENAMINE		α-NAPHTHYLMETHYL ETHYL β-CHLOROETHYL AMINE		DIMETHYL β-CHLORO β-PHENYLETHYL AMINE	
	Dose†	Mortality	Dose	Mortality	Dose	Mortality
30	20	16/20	20	0/10	1.0	9/10
			1.0	0/10		
			0.25	7/10		
60	20	8/10				
120	20	0/10	20	0/10	1.0	10/10
			1.0	0/10		
			0.25	9/10		
240	20	2/10				

* Sodium thiosulfate 1.0 gm./kgm. administered subcutaneously 30 min. prior to adrenergic blocking agent. Dose of epinephrine 20 mgm./kgm.

† mgm./kgm.

gram/kgm. intravenously, followed by dibenamine 20 mgm./kgm. intravenously, showed pressor responses to injected epinephrine when tested 30 minutes later, but after an interval of 2 hours showed typical epinephrine reversal. Similarly, mice which received sodium thiosulfate followed by dibenamine showed no protection against the lethal effects of epinephrine when tested 30 or 60 minutes later (table 4). However, when epinephrine was given 2 and 4 hours after dibenamine in similar experiments protection was evident. This delayed epinephrine protection is manifest after an interval allowing for excretion of thiosulfate and indicates the slow transformation of dibenamine *in vivo*. Similar thiosulfate experiments with α-napthylmethyl ethyl β-chloroethyl amine and dimethyl β-chloro β-phenylethyl amine failed to show this delayed protection (table 4) and indicate a more rapid transformation *in vivo* of these compounds than of dibenamine.

The ability of thiosulfate to prevent the adrenergic blocking effect of α-napthyl-

methyl ethyl β-chloroethyl amine is limited. Thus, thiosulfate prevented the protective effect of 0.25 mgm./kgm. of this agent but did not alter the effect of larger doses (table 4). Similarly in cats, even after large doses of thiosulfate (1 gram/kgm.) adrenergic block sometimes followed the injection of dibenamine, 20 mgm./kgm., administered 5 minutes later.

Thiosulfate had no effect on the protective action of dibenamine in mice when given subsequent to the dibenamine. Likewise cats which had received dibenamine 20 mgm./kgm. showed no change in the adrenergic block when large amounts of sodium thiosulfate were given.

The duration of the protection afforded mice against the lethal effects of epinephrine was compared for 3 adrenergic blocking agents (table 5). The duration of effect of both dibenamine and α-napthylmethyl ethyl β-chloroethyl amine was similar while dimethyl β-chloro β-phenylethyl amine showed a less prolonged action.

TABLE 5

Mortality of LD_{90-100} of epinephrine administered to mice at varying intervals after adrenergic blocking agents

INTERVAL BETWEEN AGENT AND EPINEPHRINE	DIBENAMINE		α-NAPHTHYLMETHYL ETHYL β-CHLOROETHYL AMINE		DIMETHYL β-CHLORO β-PHENYLETHYL AMINE	
	Dose*	Mortality	Dose	Mortality	Dose	Mortality
30 min.	20	0/10	0.5	0/10	2 0	0/10
5 hr 30 min.	20	0/10	0.5	0/10	2.0	4/10
13 hr.	20	0/10	5	1/10	5	9/10
24 hr.	20	0/10	0 5	8/10	2 0	9/10
40 hr	20	8/10	0.5	8/10		

* mgm /kgm.

DISCUSSION. A considerable number of β-chloroethyl amines have been shown to possess adrenergic blocking activity. The specificity of structure of active agents is not striking but all are tertiary substituted β-halogenated amines in which *at least one of the substituted groups is an aromatic ring separated by at least* one C atom from the N atom. The following aromatic groups have been present in active compounds: phenyl, α-napthyl, benzhydryl, and biphenoxy (1, 2). Substitution on such aromatic groups may reduce activity by altering the ability of the compound to undergo cyclization. Comparisons of the relative potency of such compounds is rendered difficult by the fact that maximal effects are reached at different rates. However, of the agents studied thus far, the α-napthylmethyl β-halogenated ethyl amines appear the most potent.

The aromatic group may be located on the chloralkyl group in an active compound as evidenced by dimethyl β-chloro β-phenyl ethyl amine. While this compound is too cholinergic to possess therapeutic potentialities, the possibility exists that analogues in which the methyl groups were replaced by higher alkyl radicals might have less of these undesirable effects.

Evidence is presented which indicates that dibenamine is transformed slowly *in vivo*. However, α-napthylmethyl ethyl β-choroethyl amine appears to undergo more rapid transformation *in vivo* and yet has an action as prolonged as that of dibenamine. The marked insolubility of dibenamine at physiological pH may be an important factor in determining its reactivity. Because of these solubility characteristics it is probable that dibenamine has considerable lipoid affinity *in vivo*. The slow rate of transformation of dibenamine does not appear responsible for its prolonged action. Rather, there is good evidence that the union between dibenamine and the effector cell is of long duration. Thus, large doses of thiosulfate do not alter the adrenergic block once dibenamine has been given. *In vitro*, the effect of dibenamine in blocking excitatory responses of the non-pregnant rabbit uterus to epinephrine is resistant to repeated washing. The nature of this prolonged effect upon the effector cell may be due to a firm fixation at a receptor surface (perhaps analagous to atropine), or, as suggested by Nickerson and Goodman (6), to the irreversible inactivation of some hypothetical enzyme system essential for excitatory responses of effector cells to epinephrine or excitatory sympathins. Both require the additional explanation of why excitatory responses of the heart to injected epinephrine or to splanchnic stimulation persist after dibenamine (6). The implication is that adrenergic receptors of the heart differ from receptors elsewhere in muscle effectors which respond to epinephrine by excitation. This is supported by the finding that isopropyl norepinephrine, which is exclusively vasodepressor, causes excitation of the heart. Changes in heart rate may thus fail to be purely a measure of the release of excitatory sympathin.

SUMMARY

1. A series of β-chloroethyl amines has been studied for evidence of adrenergic blocking activity by their ability to protect mice against lethal doses of epinephrine. Active agents were tested for the production of adrenergic block in cats.

2. From data reported herein and elsewhere, it appears that activity resides in tertiary β-halogenated amines substituted with certain aromatic groups separated by at least one C atom from the N atom.

3. Evidence is presented that dibenamine is transformed slowly *in vivo*. This does not appear to be responsible for the prolonged action of this and related compounds.

4. The absence of an effect of such adrenergic blocking agents upon the excitatory response of the heart to epinephrine is confirmed using the papillary muscle technique.

REFERENCES

1. NICKERSON, M., AND GOODMAN, L.: Fed. Proc., 5: 194, 1946.
2. LOEW, E. R., AND MICETICH, A.: Fed. Proc., 6: 351, 1947.
3. NICKERSON, M., NOMAGUCHI, G., AND GOODMAN, L.: Fed. Proc., 5: 195, 1946.
4. CATTELL, McK., AND GOLD, H.: THIS JOURNAL, 62: 116, 1938.
5. HUNT, C. C., AND PHILIPS, F. S.: THIS JOURNAL, to be published.
6. NICKERSON, M., AND GOODMAN, L.: THIS JOURNAL, 89: 167, 1946.
7. CLARK, D.: Personal Communication.
8. GILMAN, A., PHILIPS, F. S., AND KOELLE, E. S.: Am. J. Physiol., 146: 348, 1946.

THE ANTIDIURETIC EFFECT OF 3-HYDROXY-CINCHONINIC ACID DERIVATIVES[1]

E K MARSHALL, JR AND K C BLANCHARD

Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University

Received for publication October 27, 1948

The present communication presents data on the antidiuretic effect of certain derivatives of cinchoninic acid A number of these same cinchoninic acid derivatives have been found to cause an increased excretion of uric acid in man (1, 2)

METHODS The antidiuretic effect of the compounds was tested on a water diuresis in the dog Female dogs were used These had been trained and were accustomed to the passage of a stomach tube, catheterization, and venipuncture Several of the animals were subjected to perineotomy in order to facilitate catheterization Diuresis was produced by giving about 40 cc of water per kilogram by mouth Usually, urine was obtained by catheter every fifteen minutes After diuresis had been established an intravenous injection of one of the compounds was given and the effect on diuresis noted Precautions were taken to avoid the inhibition of water diuresis produced by emotional stress or afferent nerve stimulation (3, 4, 5)

All compounds were first given in a dose of 20 milligrams per kilogram, a solution of the sodium salt was used If effective, smaller doses were then used All compounds have been tested on at least two dogs

Creatinine clearance was determined in the usual manner Creatinine was given subcutaneously about one half hour and water one half and one hour before starting clearance measurements Creatinine was determined by the Folin method (6) Clearance periods varied from 10 to 22 minutes in length

PREPARATION OF COMPOUNDS All of the cinchoninic acid derivatives other than the 2 phenyl derivative (cinchophen) were prepared in this laboratory Quininic acid (6 methoxycinchoninic acid) was obtained by saponification of ethyl quininate kindly supplied by Dr Joseph B Koepfli New methods for the synthesis of 3 hydroxy 2 methylcinchoninic acid and of 3 hydroxy 2 phenylcinchoninic acid were devised to facilitate the preparation of these substances in quantity These methods are described below The remainder of the cinchoninic acids were prepared by established methods as follows 2 hydroxy 3 phenylcinchoninic acid by the method of Gysae (7), 2 hydroxycinchoninic acid by the method of Borsche and Jacobs (8), 2 hydroxy 3 methylcinchoninic acid by the method of Meyer (9), 2 methylcinchoninic acid by the method of Pfitzinger (10) and both quinaldine 3,4 dicarboxylic acid and quinoline 2,3,4 tricarboxylic acid by the method of Mitchovitch (11)

3 *Hydroxyquinaldine 4 carboxylic Acid* This substance has previously been prepared by diazotization of 3 aminoquinaldine 4 carboxylic acid (Berlingozzi and Marzella, 12) and by dimethylation of 3 methoxyquinaldine 4 carboxylic acid (Diethey and Thelen, 13) Isatin (73 5 gm , 0 5 mole) was added with shaking to a cold solution of 350 gm of 97 7 per cent sodium hydroxide in 1000 cc of water contained in a 3 liter flask When the isatin had dissolved, 70 gm (20 per cent in excess of the theoretical amount) of technical acetol acetate were added and the mixture was heated under reflux in an actively boiling water bath for 6 hours, then cooled to room temperature by immersion of the reaction flask in running water Concentrated hydrochloric acid (approximately 600 cc) was then added until precipitation of the product began, then 100 cc of glacial acetic acid were added After standing overnight at room temperature, the product was collected on a 150 mm Buchner funnel, washed with four 75 cc portions of water and sucked as dry as possible The crude product was suspended in 1200 cc of water, brought into solution by the addition of 40 cc

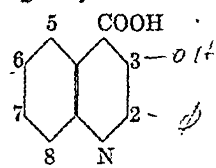
[1] This investigation has been aided by a grant from the U S Public Health Service

of 28 per cent ammonia water and the resulting solution filtered. To the filtrate, 110 cc. of 6 *N* acetic acid were added slowly with continuous stirring. After several hours at room temperature, the product was collected on a Buchner funnel, washed with six 50 cc. portions of water, sucked as dry as possible, air dried for a few days and then dried to constant weight at 60°. Yield, 91.4 gm. (90 per cent of the theoretical yield) of buff colored microcrystalline powder, m. pt. 204–206° with gas evolution.

3-Hydroxy-2-phenylcinchoninic Acid. This substance has previously been obtained by the Pfitzinger condensation of isatin with phenacyl bromide. In our experience, the substitution of phenacylacetate for the halide and the use of less concentrated alkali for the conduct of the condensation results in markedly enhanced yields of cleaner material than s obtained by the previously published methods of synthesis (14, 15).

TABLE 1

Antidiuretic effect of Cinchoninic acids



NO.	SUBSTITUENTS	EFFECT
1	2-methyl-3-hydroxy	+
2	2-hydroxy-3-methyl	–
3	2-methyl	–
4	2-methyl-3-carboxy	–
5	2-hydroxy	–
6	6-methoxy	–
7	2-3-dicarboxy	–
8	2-phenyl-3-hydroxy	++
9	2-hydroxy-3-phenyl	–
10	2-phenyl	–

The minimal quantity of a solution of 83 gm. of 97.7 per cent sodium hydroxide in 275 cc. of water necessary to effect solution was added to a suspension of 73.5 gm. (0.5 mole) of isatin in 600 cc. of water contained in a 3 liter flask. To the solution obtained, there was added first, a solution of 90.8 gm. (0.51 mole) of phenacyl acetate in 500 cc. of warm ethanol and then the remainder of the solution of sodium hydroxide. The mixture was refluxed over a free flame for three hours and allowed to remain at room temperature overnight. It was then diluted with 1300 cc. of water and filtered to remove tarry material. To the filtrate with constant stirring there was added in order 165 cc. of concentrated hydrochloric acid and 55 cc. of glacial acetic acid. After remaining at room temperature overnight, the precipitated product was collected upon a 150 mm. Buchner funnel, washed with four 50 cc. portions of cold water and transferred to a 4 liter beaker containing 1500 cc. of water. The solid was brought into solution by the addition of 40 cc. of 28 per cent ammonia water and filtered from a slight amount of insoluble matter. To the filtrate 100 cc. of 6 N acetic acid were added and, after several hours at room temperature, the product was separated by filtration, washed with four 75 cc. portions of water, dried at room temperature for a few days and then at 60° until constant weight was attained. Yield, 108.7 gm. (82 per cent of the theoretical) of deep yellow microcrystalline product, m. pt. 206–207° with decomposition.

RESULTS. Ten cinchoninic acid derivatives were tested. Results are given in table 1. Only two of the compounds exhibit an antidiuretic effect in an intra-

venous dosage of 20 milligrams per kilogram Of these two, the phenyl derivative appears to be more potent than the methyl derivative Data of experiments with three doses of the 3 hydroxy-2-phenylcinchoninic acid are given in figure 1 In figure 2, data on the effect of the same compound are given, here rate of urine flow was measured every two minutes

The relation of rate of urine flow and creatinine clearance for the two active compounds is shown in table 2 The creatinine clearance appears to be unchanged during the periods of lowest rate of urine flow

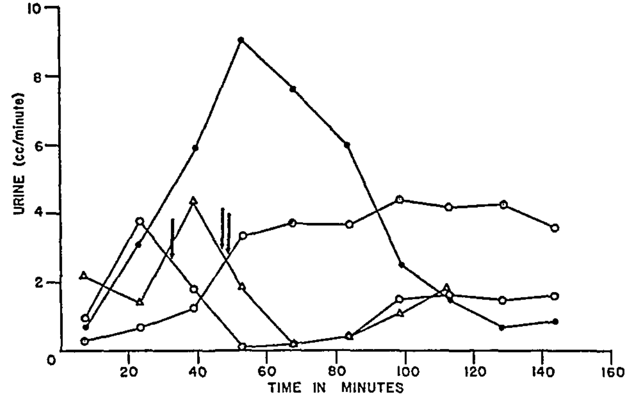


FIG 1 EFFECT OF 3 HYDROXY 2 PHENYLCINCHONINIC ACID ON WATER DIURESIS IN DOG—BROWNHEAD HOUND, 17 5 KGM

At zero minutes, 40 cc of water per kgm given by mouth Rate of urine flow plotted at middle of collection period At arrows drug given intravenously on different days —●——●— control, no drug, —O——O— 20 mgm /kgm , —△——△— 8 mgm /kgm , —O——O— 3 mgm /kgm

Experiments were done to test the efficacy of oral administration of one of the drugs Figure 3 gives the data of one of these experiments

The 3 hydroxy-2 phenylcinchoninic acid has been tested on two "neurohypophysectomized" dogs In two experiments on one dog a marked anti diuretic effect on a water diuresis was obtained, in two experiments on the other dog a slight antidiuretic effect was observed We are not reporting the results in detail because complete lack of posterior hypophyseal tissue has not yet been confirmed on these dogs and because further experiments on "neuro hypophysectomized" dogs are in progress This derivative has also been found to produce a definite reduction in the polyuria and polydipsia when given by mouth to several cases of diabetes insipidus[2]

[2] We wish to thank Dr Richard de Bodo of the Department of Pharmacology, New York University, for performing these preliminary experiments on his "neurohypophysecto mized" dogs Three cases of diabetes insipidus were studied here We are indebted to Dr Stanley E Bradley of the Presbyterian Hospital for giving the drug to other cases These studies are being continued and will be reported in detail later

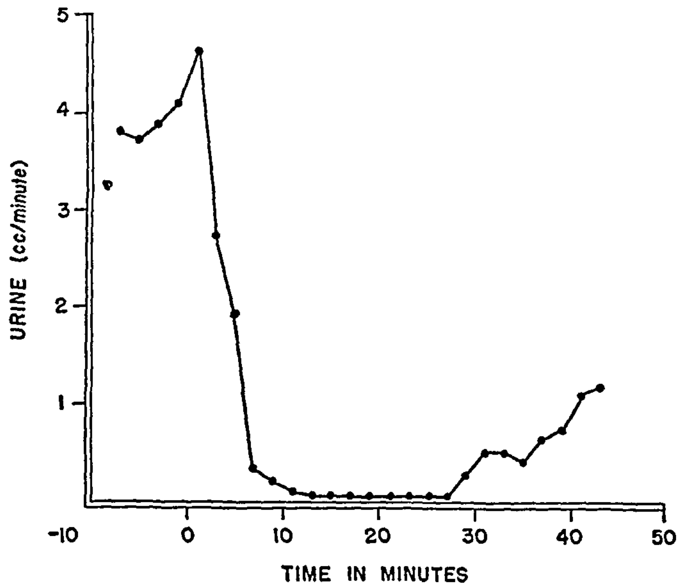


FIG. 2. EFFECT OF 3-HYDROXY-2-PHENYLCINCHONINIC ACID ON WATER DIURESIS IN DOG—SPOT, 12 KGM.

Observations started one hour after administration of 40 cc. water per kilogram by mouth. Urine flow recorded every two minutes. At zero minutes, intravenous injection of 3 mgm./kgm. of drug.

TABLE 2

Urine flow and creatinine clearance

DRUG* NO.	DOSE		1	2	3	4	5	6	7
	mgm./kgm.								
1	20	Urine Flow†	1.17	2.45	1.78‡	0.36	0.44	1.30	2.50
		Clearance†	121	107	97	102	105	104	102
1	10	Urine Flow	7.90	7.21	2.32‡	0.62	2.78		
		Clearance	103	92	80	95	101		
8	10	Urine Flow	8.20	8.00	0.78‡	0.40	0.38		
		Clearance	122	97	60	89	91		
8	10	Urine Flow	0.85	2.57	3.57	1.06‡	0.20	0.44	
		Clearance	60	75	76	47	69	68	

* See Table 1.

† Both rate of urine flow and creatinine clearance are expressed as cc. per minute.

‡ Drug injected intravenously just before start of this period.

DISCUSSION. An inhibition of water diuresis may be caused by either a decrease of glomerular filtrate (due generally to circulatory changes) or an increased reabsorption of water by the tubule. The first type of inhibition is usually rapid in onset and of short duration, while the second type is slower in onset and of longer duration than the first (5). This suggests that the inhibition

caused by the cinchoninic acid derivatives belongs to the second type. That this is so is shown by an examination of the creatinine clearances. If it is assumed that the creatinine clearance measures glomerular filtrate in the dog, it is clear that the rate of glomerular filtration is not appreciably changed during the inhibition of diuresis caused by the drug. However, a decrease in the creatinine clearance may occur in the first period after intravenous injection of drug; this occurs before the most marked decrease in rate of urine flow, and may be due to a secondary transient vascular effect of the drug.

A number of drugs besides posterior pituitary extract have been found to inhibit water diuresis. Examples are: β-imidazoylethylamine and β-oxyphenyl-

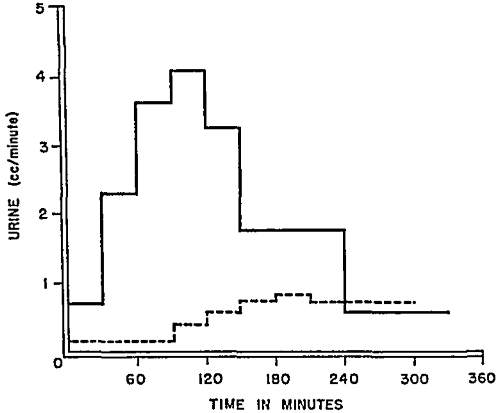


FIG. 3. EFFECT OF 3-HYDROXY-2-PHENYLCINCHONINIC ACID ON WATER DIURESIS IN DOG WHEN GIVEN ORALLY. DOG—BROWNIE, 18 KGM.

At zero minutes, 700 cc. of water by mouth. ——— control, no drug. — — — — 30 mgm./kgm. of drug given in gelatin capsules fifteen minutes before administration of water.

ethylamine (16), atropine (17, 18, 19), yohimbine (20, 21), choline (22), acetylcholine (22, 23), morphine (24), phenobarbital (25), and nicotine (26). However, of these all which have been investigated as to their mechanism of action (yohimbine, acetylcholine, nicotine, morphine and phenobarbital) appear to produce their antidiuretic effect by an action on the central nervous system causing liberation of the antidiuretic hormone of the posterior pituitary gland. The 3-hydroxy-2-phenylcinchoninic acid appears to act quite differently in that it appears to be effective in neurohypophysectomized dogs and in cases of diabetes insipidus.

SUMMARY

A number of cinchoninic acid derivatives have been examined for their antidiuretic effect in the dog. The 3-hydroxy-2-methyl and 3-hydroxy-2-phenylcinchoninic acids have an inhibitory effect on a water diuresis.

REFERENCES

1) Nicolaier, A., and Dohrn, M.: Deutsches Arch. f. klin. Med., **93:** 331, 1908.
2) Berlingozzi, S., and Donatelli, G.: Boll. Chim. Farm., **75:** 387, 1936.
3) Theobald, G. W.: J. Physiol., **81:** 243, 1934.
4) Theobald, G. W., and Verney, E. B.: J. Physiol., **83:** 341, 1935.
5) O'Connor, W. J., and Verney, E. B.: Quart. J. Exper. Physiol., **33:** 77, 1946.
6) War Department Technical Manual (TM-227): Methods for Laboratory Technicians, p. 196.
7) Gysae, G.: Ber., **26:** 2478, 1893.
8) Borsche, W., and Jacobs, W.: Ber., **47:** 354, 1914.
9) Meyer, H.: Monatsch., **26:** 1311, 1905.
10) Pfitzinger, W.: J. prakt. Chem., (2) **33:** 100, 1886.
11) Mitchovitch, V. M.: Bull. soc. Chim., (5) **6:** 1156, 1939.
12) Berlingozzi, S., and Marzello, C.: Atti accad. Lincei, (v) **32:** ii, 403, 1923.
13) Diethey, W., and Thelen, C.: Ber., **58:** 1588, 1925.
14) Berlingozzi, S., and Capuano, G. B.: Atti accad. Lincei (v) **33:** ii, 91, 1924.
15) John, H., and Fränkel, R.: J. prakt. Chem., **133:** 259, 1932.
16) Motzfeldt, K.: J. Exper. Med., **25:** 153, 1917.
17) Ginsberg, W.: Arch. f. exper. Path. u. Pharmakol., **69:** 381, 1912.
18) Cow, D.: Arch. f. exper. Path. u. Pharmakol., **69:** 393, 1912.
19) Ssargin, K. D., and Nussinboim, B. E.: Arch. internat. pharmacodyn. et de therap., **57:** 195, 1937.
20) Zunz, E., and Vesselovsky, O.: Compt. rend. Soc. de biol., **131:** 135, 1939.
21) Fugo, N. W.: Endocrinology, **34:** 143, 1944.
22) Molitor, H., and Pick, E. P.: Arch. f. exper. Path. u. Pharmakol., **101:** 198, 1924.
23) Pickford, M.: J. Physiol., **95:** 226, 1939.
24) de Bodo, R. C.: This Journal, **82:** 74, 1944.
25) de Bodo, R. C.: This Journal, **85:** 222, 1945.
26) Burn, J. H., Truelove, L. H., and Burn, I.: Brit. Med. J., **1:** 403, 1945.

γ-DICHROINE, THE ANTIMALARIAL ALKALOID OF CH'ANG SHAN[1]

FRANCIS G HENDERSON, CHARLES L ROSE, PAUL N HARRIS, AND K K CHEN

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis

Received for publication October 27, 1948

In China, for many centuries, a medicinal herb called Ch'ang Shan has been known for its antimalarial action (2–3) Its emetic and cathartic effects have also been described The root is employed in medicine, although the leaves are also believed active The above-mentioned Chinese references give directions how to make a tea, a wine, or pills of the dried root of Ch'ang Shan, for the control of various forms of malaria The plant, an evergreen shrub, is indigenous to Southwestern China After the introduction of quinine in China, Ch'ang Shan gradually became a remedy of only local interest—namely in Yunnan and Szechuan Provinces

There is much confusion about the botanical identity of Ch'ang Shan Some authors named it *Orixa japonica* (4–5), while others called it *Dichroa febrifuga* (6) Still other terminology was discussed by Yang (7) Although there has been no final verification, most investigators accept *Dichroa febrifuga* as the correct name The pharmacognosy of Ch'ang Shan and its cultivation have recently been presented by Yu (8)

Several Japanese workers became interested in Ch'ang Shan, and studied its history, histology, and geographical distribution (9–10) Terada and his co-workers failed to show the efficacy of the infusion of Ch'ang Shan in the *Plasmodium* infection of canaries (11) They, too, ran into controversy over its botanical identity (9, 12–13) Since Terasaka's chemical investigation (13) was carried out with the Japanese variety of *Orixa japonica,* his results do not apply to Ch'ang Shan

During the Japanese occupation of Eastern China in World War II, the Chinese government was forced to move to Chungking, a city in Southwestern China With it, was the migration of large masses of people from coastal and central provinces Unfortunately, Southwestern China is a pandemic area of different forms of malaria A large portion of the new population, including the governmental and military personnel, fell victims of malaria, and sustained a high mortality rate Meanwhile, the Dutch East Indies were lost to the Japanese, resulting in a cut-off of 90 per cent of the world's supply of quinine to the allies Under this desperate condition, the Chinese immediately directed their attention to crude drugs of their own, including Ch'ang Shan

Scientific studies were initiated in Chinese governmental laboratories Although the preliminary note of Liu and his associates (14) was not enlightening, their results with the crude powder in humans seemed to indicate that the herb

[1] Read in part at the Fall Meeting of the American Physiological Society at Minneapolis, Minnesota, on September 17, 1948 (1)

was efficacious in the treatment of tertian malaria. The work was continued at the National Institute of Health by Jang and his colleagues (6, 15–17), and the therapeutic effect of the extract of Ch'ang Shan in 13 clinical cases was confirmed. Progress was made when Chou and his co-workers (18–19), at the Institute of Materia Medica, Shanghai, announced the isolation of 3 isomeric alkaloids in crystalline form, of which γ-dichroine was the most active in *gallinaceum* infection of chickens.

In the United States, the Board for the Coördination of Malarial Studies became interested in Ch'ang Shan, and assigned to it the survey number SN-10,767 (20). In coöperation with the Board, our laboratories purchased in the fall of 1942 360 pounds of Ch'ang Shan from Chinese drug shops in different localities—Honolulu, San Francisco, Vancouver, B. C., Chicago, New York City, and Havana. In addition, we imported 500 pounds from Chungking, China, by an American military plane. Koepfli, Mead, and Brockman (21), using the extracts we prepared, succeeded in isolating 2 isomeric alkaloids, one of which, febrifugine, is about 100 times as active as quinine (Q value of 100) against *P. lophurae* in ducks. Kuehl, Spencer, and Folkers (22), working independently, also obtained 2 isomeric alkaloids having Q values of 8 and 16, respectively, against *P. gallinaceum* in chicks. It is interesting that the proposed empirical formula of the two groups of American investigators is the same, namely, $C_{16}H_{19}O_3N_3$. It also agrees with the formula of the Chinese workers, published in their preliminary note (18), but differs from the revised formula of their second paper (19) by 2 atoms of hydrogen.

Ch'ang Shan has been studied in England by Tonkin and Work (23) and Hooper (24). The latter separated a glucoside, but failed to find any alkaloids.

Dr. T. Q. Chou, Director of the Institute of Materia Medica, Shanghai, made available to us a generous supply of γ-dichroine. Our study was limited to the estimation of its antimalarial activity, determination of its activity, and assessment of its effects on blood pressure, respiration, gastrointestinal tract, blood sugar, and body temperature. The material was crystalline-white, was in needle-form under the microscope, and melted at 160°C. (corrected). The alkaloid required dilute acid for solution—a volume of 3.42 cc. of N/100 HCl for every 10 mgm. of γ-dichroine. For pharmacologic experiments, a fresh stock solution of 0.1 per cent was prepared.

1. Antimalarial Activity. (*a*) *Ducks.* The method of study was similar to I–2 already described (25), with certain modifications. Briefly, ducklings, 4-weeks-old, were infected with *P. lophurae* by transfusion of blood from another duckling previously infected with the same organism. The infecting dose was 2 billion parasitized red cells per kgm. of body weight. Each animal was treated with γ-dichroine by intravenous injection within the first 4 hours, and again within 8 hours, after inoculation. The medication was continued for 5 days, 3 times a day. On the sixth day, parasitized erythrocytes were counted on thin films. Control animals with quinine, and without any medication, were run at the same time. A total of 134 ducklings were used.

The results are summarized in table 1. It should be observed that ducklings

stomach tube to rhesus monkeys infected with *P. cynomolgi*. Since Dr. Schmidt will publish his results in detail, it suffices to mention that doses of γ-dichroine ranging from 0.4 to 0.8 mgm. per kgm. definitely reduced the parasitemia, and one of 1.6 mgm. per kgm. cleared the blood stream of parasites to such an extent that a thick film showed no count (26).

2. Toxicity. The acute toxicity of γ-dichroine was determined in starved albino mice by both intravenous and oral administration. These studies were made on the same day, with the same solution, and on the same group of mice. Death occurred in 48 to 72 hours. As shown in table 2, the median lethal dose (LD_{50}) by mouth is 2.74 ± 0.41, and by vein, 10.0 ± 0.50, mgm. per kgm. It is *of particular interest that this alkaloid is approximately $3\frac{1}{2}$ times as toxic when* given orally than by vein.

Repeated intravenous injections at different dose levels were given to mice in *an attempt to obtain some knowledge of any pathology which might occur.* Necropsy was performed immediately following the death of the animal. Table 3 summarizes the results of this work. Of the 27 animals which came to necropsy, one animal showed parenchymatous degeneration of the kidney with some necrosis. Twelve animals (44.4 per cent) showed hydrops of the liver characterized by large, vacuolated hepatic cells, as illustrated in figure 1. The same lesion occurred after γ-dichroine was injected subcutaneously or intraperitoneally (table 3).

TABLE 2

The acute toxicity of γ-dichroine in mice

ROUTE OF ADMINISTRATION	DOSE	NUMBER DIED / NUMBER USED	$LD_{50} \pm$ S. E.
	mgm./kgm.		*mgm./kgm.*
Intravenous	8.0	1/10	10.0 ± 0.5
	10.0	5/10	
	12.5	9/10	
	16.0	10/10	
Oral	0.625	0/10	2.74 ± 0.41
	1.25	2/10	
	2.5	2/10	
	5.0	9/10	
	8.0	10/10	

3. Emetic Action. γ-Dichroine produced vomiting in pigeons. When it was administered by vein to starved pigeons, the initial episode of vomiting occurred in 15 to 30 minutes. This was followed by repeated attacks of vomiting at 5- to 10-minute intervals. The incidence of vomiting with different doses over the number of birds used is as follows: $\frac{2}{5}$ on a dose of 0.1, $\frac{3}{5}$ on one of 0.2, and $\frac{4}{5}$ on one of 0.3, mgm. per kgm. The median emetic dose (EmD_{50}) is 0.132 ± 0.04 mgm. per kgm. It would appear that γ-dichroine is responsible for vomiting in man when Ch'ang Shan or its extract is used in the treatment of malaria (2, 3, 6, 14, 15).

4 *Effect on Respiration and Circulation* Because of the small amount of material available, studies on respiration and circulation were limited Dogs under 'Sodium Amytal' (Sodium Iso amyl Ethyl Barbiturate, Lilly) anesthesia

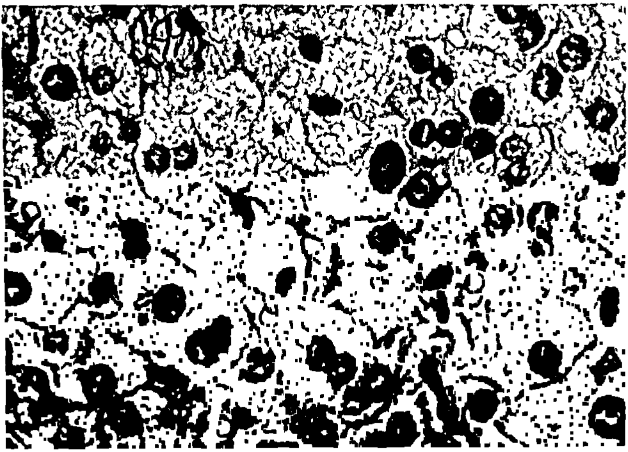


FIG 1 HYDROPIC DEGENERATION OF THE LIVER

Mouse 1, weighing 23 5 gm received a daily dose of 4 mgm per kgm of γ dichroine by intraperitoneal injection It died following the eighth dose

TABLE 3

Results of repeated injections of γ dichroine in mice

DOSE	NUMBER OF ANIMALS	AVERAGE NUMBER OF INJECTIONS PER MOUSE	ROUTE OF ADMINISTRATION	NUMBER TO NECROPSY	PATHOLOGY
mgm / kgm					
2	5	14	Intravenous	5	1 Hydrops of liver, 1 parenchymatous degeneration of kidneys
4	5	8	Intravenous	4	4 Pulmonary edema and atrophy of thymus
8	5	4	Intravenous	4	2 Hydrops of liver
10	10	2	Intravenous	5	All showed minimal or slight hydrops of liver
4	5	8	Subcutaneous	4	2 Hydrops of liver
4	5	7	Intraperitoneal	5	2 Hydrops of liver

showed a fall in blood pressure of 20 mm Hg following the intravenous injection of 4 mgm per kgm There was a transient increase in respiratory rate Smaller doses, such as one of 2 5 mgm per kgm, produced very slight changes in both blood pressure and respiration, as exemplified in figure 2

5. Action on Intestine. As shown in figure 2, a dose of 2.5 mgm. per kgm. of *γ-dichroine caused* an increase in duodenal peristalsis in anesthetized dogs. The duodenal movements were recorded by a balloon through a Harvard membrane manometer. The increase in activity began 5 to 10 minutes following injection and lasted for 2 to 3 hours. This picture was duplicated in 3 other dogs. Following decerebration and pithing, and also double vagotomy, the alkaloid produced the same stimulation of intestinal activity. With atropinization, the stimulating action of γ-dichroine was inhibited for a short time.

In 4 non-anesthetized rabbits, doses of 5 and 10 mgm. per kgm. of the alkaloid induced marked diarrhea in 30 minutes following intravenous injection, which persisted for 5 to 6 hours. Ten non-anesthetized rats also developed pronounced diarrhea—each following a dose of 2.5 mgm. per kgm., 5 by intravenous injection

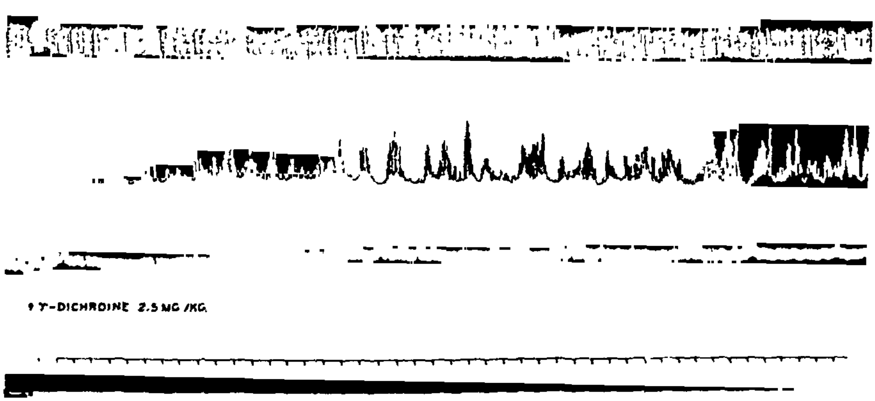


Fig. 2. Action of γ-Dichroine on Respiration, Blood Pressure, and Duodenum

Dog 6816, weighing 6.1 kgm., was anesthetized with 'Sodium Amytal', 70 mgm. per kgm. Tracings from top down are respiratory movements, duodenal movements, carotid blood pressure, time in minutes, and baseline. γ-Dichroine was injected intravenously.

and 5 by oral administration. When isolated strips of the rabbit's ileum were immersed in Locke-Ringer's solution, γ-dichroine inhibited the activity, as shown in figure 3. This action is entirely opposite to what may be expected as far as diarrhea is concerned. It is also not in agreement with the results in anesthetized dogs in which stimulation is the predominant feature of γ-dichroine. On account of the limited quantity of the alkaloid at our disposal, this phase of the study was not continued.

6. Blood Sugar. The effect of γ-dichroine on blood sugar was determined in albino rabbits. Fasting blood sugars were determined by the method of Hagedorn and Jensen (27), and the alkaloid was given by vein. Blood samples were taken after injection at 15 and 30 minutes, and 1, 2, 3, 4, 5, and 6 hours. Doses of 5 and 10 mgm. per kgm. in 4 rabbits gave very erratic blood sugar curves which can best be explained by the severe diarrhea produced, as mentioned above. A dose of 2.5 mgm. per kgm. in 3 additional rabbits caused a rise of

blood sugar, reaching a maximum in 3 to 4 hours. This dose was not followed by diarrhea. Figure 4 illustrates the blood sugar changes of these 3 animals.

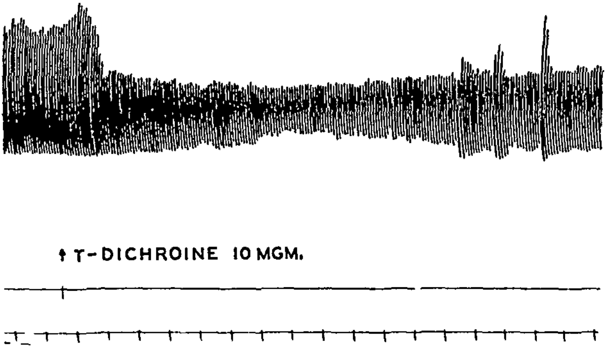


FIG 3 ACTION OF γ DICHROINE ON THE ISOLATED RABBIT'S ILEUM
The final concentration of γ dichroine in the bath was 1:10,000. Note the inhibition of peristalsis which lasted more than 15 minutes

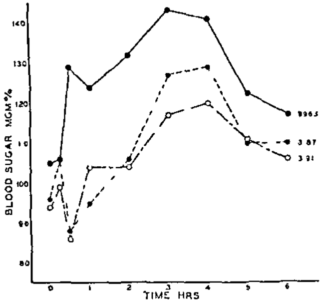


FIG 4 ACTION OF γ-DICHROINE ON BLOOD SUGAR
The sexes and body weights of the 3 rabbits were as follows No 9963, female, 2 085 kgm ; No 3187, female, 1 890 kgm , and No 3191, female, 1 875 kgm Each animal received γ dichroine in the dose of 2 5 mgm per kgm intravenously

7. *Antipyretic Action.* Previous investigation by Liu and his co-workers (14) revealed that the extract of Ch'ang Shan had an antipyretic action. It was

thus desirable to test the alkaloid for this action. The method of Smith and Hambourger (28) was employed. Briefly, albino rats were injected subcutaneously with a yeast suspension 18 hours prior to the test period. Rectal temperatures were taken by the use of a thermocouple. Acetylsalicylic acid was tested simultaneously and used as a standard for antipyretic activity. As shown in figure 5, γ-dichroine in a dose of 2.5 mgm. per kgm. orally in 5 rats has an antipyretic activity of greater magnitude and longer duration than acetylsalicylic acid. The dose of the latter was 25 mgm. per kgm., and the number of rats used was also 5.

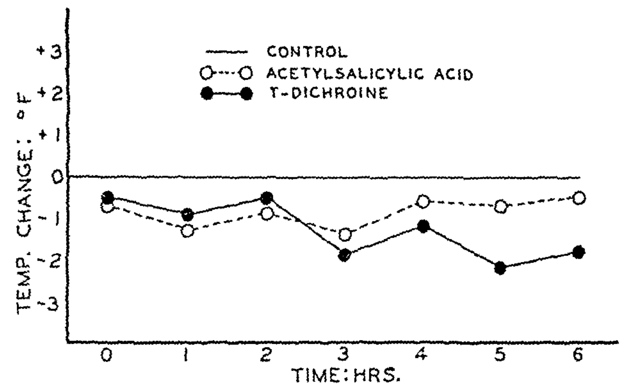


FIG. 5. THE ANTIPYRETIC ACTION OF γ-DICHROINE

Each curve represents the average differences of rectal temperature of a group of 5 rats from that of a control group of 5 rats, which occupies the straight line. All animals received a yeast suspension 18 hours prior to the temperature readings.

DISCUSSION. γ-Dichroine is unquestionably a potent antimalarial alkaloid in birds. It is also effective in monkey malaria, and, in all probability, is responsible for the activity of Ch'ang Shan or its extract in human malaria. These results substantiate the observations made by the Chinese many centuries ago. It is doubtful, however, that γ-dichroine can replace quinine, chloroquine, or other antimalarial agents, because it tends to cause nausea, vomiting, diarrhea, and hydropic degeneration of the liver in animals. Furthermore, it will be extremely costly to manufacture the alkaloid in large quantities from the root of the plant. A better plan is to synthesize and investigate the derivatives of quinazoline, since the latter is a degradation product of γ-dichroine (19).

Whether or not γ-dichroine is identical with febrifugine (21) and alkaloid II of Kuehl and his associates (22) will require further investigation. The Chinese and American chemists arrived at about the same composition by combustion analysis. There is a definite discrepancy, however, in their physical constants. All three groups obtained isomeric alkaloids of lower antimalarial potency. Direct comparison of the samples from the 3 laboratories is in order.

It is curious that γ-dichroine, in contrast with most other drugs, is roughly $3\frac{1}{2}$ times as toxic to mice orally as intravenously. No adequate explanation is on hand. Either elimination is extremely rapid by vein, or decomposition takes place in the gastrointestinal tract, and the degradation products are

especially toxic to the animal. In our laboratory, it was previously noted that the soluble derivatives of 4,4-diamino-1,1-diphenyl sulfone were also more toxic by mouth than by vein (unpublished data).

For want of material, several questions were left completely unanswered. The stimulating action of γ-dichroine on the dog's duodenum and the inhibiting action on the isolated rabbit's ileum need further exploration. Although the emetic action following intravenous injection is probably central in origin, it deserves more work to exclude the local action. It is also desirable to determine how the hyperglycemia is brought about.

SUMMARY

1. γ-Dichroine, an alkaloid of Ch'ang Shan, has a Q value of 148 against *Plasmodium lophurae* in ducklings, and a Q value of 137 against *P. relictum* in canaries. It is also active against *P. cynomolgi* in monkeys.

2. γ-Dichroine in a single dose is approximately $3\frac{1}{2}$ times as toxic to mice by mouth than by vein. Repeated administration of γ-dichroine in mice may result in hydropic degeneration of the liver.

3. γ-Dichroine induces vomiting in pigeons following intravenous injection, the $EmD_{50} \pm$ S. E. being 0.132 ± 0.04 mgm. per kgm.

4. γ-Dichroine has no effect on blood pressure and respiration in anesthetized dogs in the dose of 2.5 mgm. per kgm., but slightly lowers blood pressure with an acceleration of the respiratory rate in the dose of 4 mgm. per kgm.

5. γ-Dichroine stimulates duodenal peristalsis in anesthetized dogs, but inhibits the activity of the isolated rabbit's ileum. It causes diarrhea in non-anesthetized rabbits and rats following intravenous injection.

6. γ-Dichroine produces hyperglycemia in rabbits in the dose of 2.5 mgm. per kgm. when injected intravenously. In rats, the same dose when given by mouth shows a slightly greater antipyretic action than acetylsalicylic acid.

Acknowledgment. The authors are indebted to Messrs. C. E. Powell, H. M. Worth, and J. H. Wheeler for their invaluable assistance in many of the experiments.

REFERENCES

1. CHEN, K. K.: Fed. Proc., (in press).
2. LI, S. C.: Pentsao Kang Mu, chapter 17, part A, 1596.
3. CHEN, C. J.: Chinese Dispensatory, 2: 1143, Global Press, Shanghai, 1935.
4. STUART, G. A.. Chinese Materia Medica, 292, American Presbyterian Mission Press, Shanghai, 1911.
5. BOTANICAL NOMENCLATURE, 885, Commercial Press, Shanghai, 1918.
6. JANG, C. S.: Chin. Med. J., 62: 185, 1944.
7. YANG, S. T.: J. Am. Pharm. A., Scient. Sect., 37: 458, 1948.
8. YU, T. C.: J. Chin. Pharm. A., 7: 24, 1948.
9. KIMURA, K.: J. Pharm. Soc. Japan, 48: 884, 1928.
10. NAKAO, M.: J. Pharm. Soc. Japan, 48: 893, 1928.
11. TERADA, Y., SUZAKI, K., NAMURA, T., AND TADA, E.: Nagoya Igakkai Zasshi, 54: 43, 1941; through Chem. Abst., 42: 724, 1948.
12. KIMURA, K., AND SHIMADA, H.: J. Pharm. Soc. Japan, 48: 896, 1928.
13. TERASAKA, M.: J. Pharm. Soc. Japan, 51: 707, 1931.

14. Liu, S. K., Chang, Y. T., Chuan, T. K., and Tan, S. C.: Nat. Med. J. China, **27**: 327, 1941; Chin. Med. J., **59**: 575, 1941.
15. Jang, C. S., and Chou, T. C.: Nat. Med. J. China, **27**: 137, 1943.
16. Jang, C. S., Fu, F. Y., Wang, C. Y., Huang, K. C., Lu, G., and Chou, T. C.: Science, **103**: 59, 1946.
17. Jang, C. S., Fu, F. Y., Huang, K. C., and Wang, C. Y.: Nature, **161**: 400, 1948.
18. Chou, T. Q., Jang, C. S., Fu, F. Y., Kao, Y. S., and Huang, K. C.: Science (Chinese), **29**: 49, 1947.
19. Chou, T. Q., Fu, F. Y., and Kao, Y. S.: J. Am. Chem. Soc., **70**: 1765, 1948.
20. Wiselogle, F. Y.: A Survey of Antimalarial Drugs, **2**: 1603, 1946.
21. Koepfli, J. B., Mead, J. F., and Brockman, J. A., Jr.: J. Am. Chem. Soc., **69**: 1837, 1947.
22. Kuehl, F. A., Jr., Spencer, C. F., and Folkers, K.: J. Am. Chem. Soc., **70**: 2091, 1948.
23. Tonkin, I. M., and Work, T. S.: Nature, **156**: 630, 1945.
24. Hooper, D.: Nature, **157**: 106, 1946.
25. Wiselogle, F. Y.: A Survey of Antimalarial Drugs, **1**: 485, 1946.
26. Schmidt, L. H.: Private communication.
27. Hagedorn, H. C., and Jensen, B. N.: Biochem. Ztschr., **135**: 46, 1923.
28. Smith, P. K., and Hambourger, W. E.: This Journal, **54**: 346, 1935.

HEINZ BODY FORMATION BY CERTAIN CHEMICAL AGENTS

STEWART H WEBSTER, ERVIN J. LILJEGREN AND DAVID J ZIMMER

Laboratory of Physical Biology, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Maryland

Received for publication October 28, 1948

INTRODUCTION Heinz bodies, also known as Heinz-Ehrlich-, inner- or inclusion bodies, are refractile granules or particles sometimes found in erythrocytes [1] These bodies were named after the pharmacologist, Robertz Heinz, who in 1890 (2) discovered them in the red blood cells of animals poisoned by certain aromatic compounds In general, such formation was limited to phenylhydrazine and its derivatives, as well as to some aromatic amino and nitro compounds, although hydroxylamine and chlorates were found to have similar action The work of Moeschlin and Hurschler (3–7) on the formation of Heinz bodies both *in vivo* and *in vitro* by certain sulfonamides caused renewed interest in the subject Since the initial work of Heinz, more than a hundred investigations have been carried out in the attempt to elucidate the chemical nature of Heinz bodies, to account for their mechanism, and to devise methods for staining and differentiating them from similar particles

During an investigation of the effect of stibine (antimony hydride, SbH_3) on experimental animals, the presence of small refractile granules was occasionally noted in the erythrocytes of these subjects These granules were first observed by us in mice and guinea pigs and later in other species and it was initially assumed that they were Heinz bodies In the course of work with other hemolytic and chemical substances similar inclusion bodies were noted This research was undertaken in order to investigate their behavior and to establish their identity Due to the exploratory nature of the study, fewer animals were used in testing each substance than would be necessary for statistical evaluation of the data Since this work was incidental to the stibine study, to be reported elsewhere, further work is not contemplated at the present time

METHODS OF OBSERVATIONS OF HEINZ BODIES Using wet preparations of blood, the small, colorless refractile granules in the erythrocytes were stained blue with methyl violet dye, in the manner suggested by Heinz (2)

As used in this work, 2 93 grams of methyl violet dye (C I 680 dye content 88 per cent) is shaken with 100 cc of 0 6 per cent sodium chloride solution, filtered and diluted with an equal volume of 0 85 per cent NaCl This results in an approximately half saturated solution of the dye in 0 73 per cent NaCl In use, a drop of this solution is placed on a slide, and covered with a slip containing a small drop of blood on the bottom surface After standing for 2–3 minutes the staining of any Heinz bodies present should be nearly complete Observations under high power (1400–1500 ×) are best made on thinner fields where the red cells are sufficiently separated to enable counts to be carried out Using critical illumination and by focusing up and down, blue particles as small as a fraction of a

[1] A review of the extensive literature on Heinz body phenomenon has been given elsewhere (1)

micron can be seen, the rolling of the cells during movement facilitating this observation. Using this technique, the occurrence of the so-called Rand bodies (8), which might be confused with Heinz bodies, was only rarely observed. Almost without exception the Heinz bodies were found only in mature red cells, the reticulocytes only rarely showing these particles.

Usually 8 or 10 fields were examined for the presence of Heinz bodies if few were seen. However, when these were numerous a count was made, usually 100 or 200 cells being carefully studied. The number of red blood cells having one or more blue particles was determined and a rating was made in accordance with the following scheme:

Percentage showing Heinz bodies	*Evaluation*
Occasional	±
1–10%	+
11–40%	2+
41–75%	3+
76–100%	4+

During the investigation of this phenomenon a new technique for staining these granules in smears was worked out (9). This involved treatment of the fresh air-dried smears with 0.2 per cent methyl violet in 95 per cent ethyl alcohol for one-half minute. It was found that preliminary fixation with methyl alcohol resulted in shedding of many of the particles from the erythrocytes and these could be seen on the slide. With the new technique the granules were fixed sufficiently to hold them within the cells while they were being stained. Some of the granules, however, were removed initially from the cells through trauma produced by the smearing operation. For these reasons the number of intraerythrocytic particles was much greater in the wet preparations than it was in the fixed slides. Hence the former method was preferred except for photographic purposes. By means of a special technique, using electronic photoflash equipment (10), photomicrography of Heinz bodies in wet preparations was found to be practicable.

Experimental procedures. A number of chemical substances were used experimentally in order to investigate the production of so-called inclusion bodies in the erythrocytes, several kinds of experiments being tried.

In the experiments made *in vivo*, stibine, a volatile substance, was administered to various species of animals in exposure chambers at predetermined concentration levels. Ordinarily a single one-hour exposure was used but with higher concentrations the time was often shortened. Sulfanilamide was given either by intraperitoneal injection of an aqueous solution, or of a suspension in gum acacia, or by using a 0.3 per cent solution of the material in the drinking water. Aqueous solutions of the remaining substances were ordinarily used for intraperitoneal injection, the dosage being calculated in terms of mgm./kgm. body weight.

The animals used in these experiments were drawn from the stock colony of the National Institutes of Health and only those in apparent good health were used. Care was taken to test the blood of the animals to be used experimentally and reject those showing more than an occasional inclusion body (± rating). The white mice used were of the N.I.H. strain, derived in turn from the white Swiss variety.

Finally, a few *in vitro* experiments were conducted using freshly shed mouse or guinea pig blood. One cc. of blood was mixed with 25 cc. of solution composed of 7 vol. of Locke's solution (11) to 3 vol. of water and containing the toxic substance in the desired concentration. After testing blood from tail or ear for Heinz bodies, the animal was bled and the blood was mixed immediately with the solution. The mixture was incubated at 37°C. and tests were made on the red cells at various intervals thereafter in the manner described.

Action of stibine. Using wet blood preparations, it was observed that refractile granules appeared in mature erythrocytes of mice immediately follow-

ing a single one-hour exposure to stibine at a concentration level of 70 p.p.m. With a guinea pig given a similar exposure, the granules appeared after a few hours. Within six hours, 90 per cent and 75 per cent of the mouse and guinea pig cells, respectively, showed similar alterations.

Continuous observation over a period of 24 hours was carried out on 20 guinea pigs exposed to 53 p.p.m. of stibine for 1 hour. Hematological examinations were made immediately following exposure and every 4 hours thereafter. One animal died shortly after the end of the exposure. Of the remaining 19 animals, 4+ inner bodies were shown by 16 within 8 hours and by all within 12 hours. In 2 animals one or more tiny particles were present in small numbers within 4 hours. Blood destruction was evidenced not only by hemolysis and drop in red count but by hemoglobinuria in all but one animal. The inner bodies were present in the blood of all guinea pigs when they were sacrificed at the end of 3 days, varying in amounts from ± (1 animal) to 4+ (7 animals). Growth in the size of the particles over the three-day period was quite evident. Similar results were noted in another series of 25 guinea pigs, having a single exposure to stibine, all of the animals showing the presence of intraerythrocytic particles when examined 24 hrs. after exposure. Fig. 1 shows the typical appearance of inner bodies in erythrocytes from a guinea pig 4 days after exposure of the animal to stibine.

The rat is apparently more resistant, for the bodies appeared later and were less numerous than in the other species. The granules were observed also in rabbits, cats, dogs, and monkeys.

The bodies were found to persist for relatively long periods of time. In mice they were observed for 55 days, in a rat for 33 days and in a guinea pig for 11 days following a single exposure to stibine. These granules stained well with methyl violet and gentian violet, both supravitally and in smears, and they were insoluble in water, dilute acetic acid and ethyl and methyl alcohols. Their sizes varied with the species, the particles usually being much smaller in guinea pigs and rabbits than in mice. Furthermore, the particles had all the morphologic characteristics described by Heinz in his original communication (2). It seems evident, therefore, that the bodies produced by stibine are identical with the Heinz bodies described so many years ago.

Attempts were made to produce Heinz bodies *in vitro* by passing stibine through heparinized blood of various species of animals. Although morphologic changes were evidenced by the formation of spine cells with guinea pig blood, no Heinz bodies were observed in any case.

Action of sulfanilamide. Using the method of Figge (12), Heinz bodies were produced in mice given a 0.3 per cent solution of sulfanilamide for their drinking water. In less than 24 hours the presence of blue-staining refractile granules could be observed when using the supravital technique mentioned above. Fig. 2 shows the appearance of the Heinz bodies, using the electronic photoflash technique (10) to stop the motion of the cells in the supravital preparation. At first the particles appeared as tiny strands or dots, many of which exhibited Brownian motion, and even the smallest ones could be easily seen as

the cells rolled over and came into focus. These intraerythrocytic bodies were found to stain well with methyl violet and in both morphologic and chemical behavior they exhibited the properties of Heinz bodies.

It was characteristic that as these particles developed they became larger and more nearly round in shape and at times they appeared to protrude and were attached to the cell by a thick membrane. Only rarely were the Heinz bodies observed leaving the cells but apparently they do so, since the particles were seen outside the cells and they were easily extruded on smearing blood containing these granules. Fig. 3 illustrates this extrusion of Heinz bodies.

Intraperitoneal injection of sulfanilamide in mice at levels of 1000 mgm./kgm., comparable to that used in the above experiments, resulted in Heinz bodies in the erythrocytes 2–3 hrs. after injection, a + and 2+ response being observed in 2 animals after 24 hrs.

The chronic effect of sulfanilamide, with prolonged Heinz body formation, was studied by administering 0.3 per cent solution of this drug as drinking water to white mice for a period of 6 months. The experimental group consisted of 3 males, 2 additional males being used as controls. In the first month, during the cleaning of the nests, one of the male test animals was inadvertently replaced by a female. This resulted in pregnancy so that it was of interest to study the offspring. During the test period 6 litters, or a total of 35 mice, were born to this mother. All of the young appeared to be in good health and Heinz bodies were not present in the newly born mice during the suckling period. An experiment in which sulfanilamide crystals were implanted in a suckling and in a mature mouse resulted in finding Heinz bodies in the blood stream of the mature but not of the young animal. Intraperitoneal injection of another pair of mice with the same substance (1000 mgm./kgm.) resulted in a maximum response of + (1 per cent) and 2+ (16 per cent) for the suckling and the mature animal, respectively.

On supravital biweekly examinations, Heinz body estimations throughout the 183-day period were always 4+ on all of the treated mice, the controls being negative during the entire period. During the later stages the Heinz bodies were very large, exhibiting poor to moderate staining qualities. Numerous erythrocytes were seen to have more than one inclusion body.

Before sacrificing the animals at the end of the six-month (183-day) period blood was taken and urine was secured from the bladder of each animal. No hemoglobin was found in any urine sample. Blood from the female mouse appeared to be quite normal with the exception of finding 4+ Heinz bodies. The liver and kidneys of this animal were normal but the spleen was very black and large. The data for the male test group are summarized in table 1.

The organ: body weight ratios for livers and kidneys did not differ greatly in the treated and the control groups; the spleens of the treated animals, although having higher ratios than those for the control group, were below the average found for animals of this weight (13) and hence did not indicate enlargement.

The histopathologic examination revealed no significant changes in the hearts or lungs of the test animals. The spleens showed 2+ to 4+ iron, ± iron

in an occasional convoluted tubule and + iron in the Kupffer cells of the liver of one animal

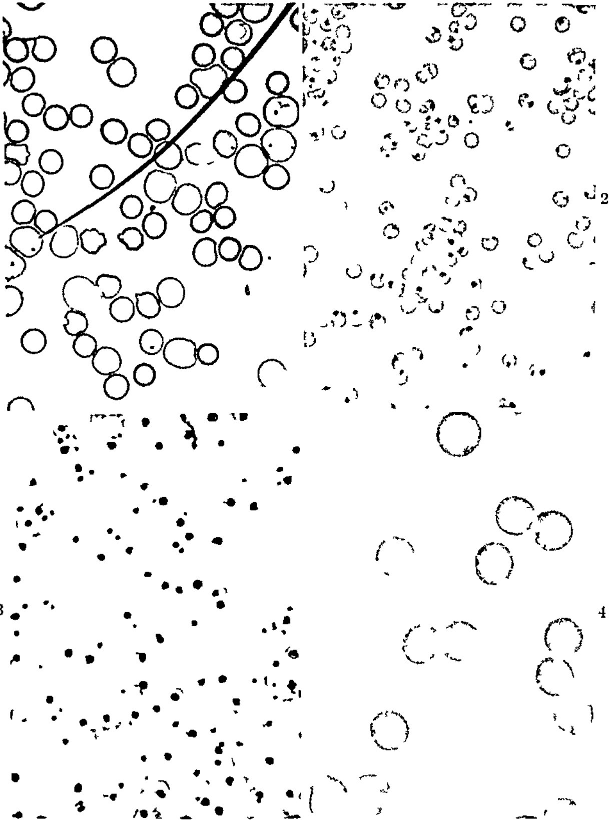


FIG 1 HEINZ BODIES FORMED IN VIVO BY STIBINE SMEAR OF GUINEA PIG BLOOD × 660
FIG 2 HEINZ BODIES IN WET PREPARATION OF MOUSE BLOOD × 660
FIG 3 EXTRUSION OF HEINZ BODIES STAINED SMEAR OF MOUSE BLOOD × 660
FIG 4 IN VITRO FORMATION OF HEINZ BODIES BY PHENYLHYDRAZINE WET PREPARATION OF GUINEA PIG BLOOD × 1350

From the data given it will be noted that the prolonged ingestion of sulfanilamide had little influence on weight, red cell count, hemoglobin or hematocrit

the cells rolled over and came into focus. These intraerythrocytic bodies were found to stain well with methyl violet and in both morphologic and chemical behavior they exhibited the properties of Heinz bodies.

It was characteristic that as these particles developed they became larger and more nearly round in shape and at times they appeared to protrude and were attached to the cell by a thick membrane. Only rarely were the Heinz bodies observed leaving the cells but apparently they do so, since the particles were seen outside the cells and they were easily extruded on smearing blood containing these granules. Fig. 3 illustrates this extrusion of Heinz bodies.

Intraperitoneal injection of sulfanilamide in mice at levels of 1000 mgm./kgm., comparable to that used in the above experiments, resulted in Heinz bodies in the erythrocytes 2–3 hrs. after injection, a + and 2+ response being observed in 2 animals after 24 hrs.

The chronic effect of sulfanilamide, with prolonged Heinz body formation, was studied by administering 0.3 per cent solution of this drug as drinking water to white mice for a period of 6 months. The experimental group consisted of 3 males, 2 additional males being used as controls. In the first month, during the cleaning of the nests, one of the male test animals was inadvertently replaced by a female. This resulted in pregnancy so that it was of interest to study the offspring. During the test period 6 litters, or a total of 35 mice, were born to this mother. All of the young appeared to be in good health and Heinz bodies were not present in the newly born mice during the suckling period. An experiment in which sulfanilamide crystals were implanted in a suckling and in a mature mouse resulted in finding Heinz bodies in the blood stream of the mature but not of the young animal. Intraperitoneal injection of another pair of mice with the same substance (1000 mgm./kgm.) resulted in a maximum response of + (1 per cent) and 2+ (16 per cent) for the suckling and the mature animal, respectively.

On supravital biweekly examinations, Heinz body estimations throughout the 183-day period were always 4+ on all of the treated mice, the controls being negative during the entire period. During the later stages the Heinz bodies were very large, exhibiting poor to moderate staining qualities. Numerous erythrocytes were seen to have more than one inclusion body.

Before sacrificing the animals at the end of the six-month (183-day) period blood was taken and urine was secured from the bladder of each animal. No hemoglobin was found in any urine sample. Blood from the female mouse appeared to be quite normal with the exception of finding 4+ Heinz bodies. The liver and kidneys of this animal were normal but the spleen was very black and large. The data for the male test group are summarized in table 1.

The organ: body weight ratios for livers and kidneys did not differ greatly in the treated and the control groups; the spleens of the treated animals, although having higher ratios than those for the control group, were below the average found for animals of this weight (13) and hence did not indicate enlargement.

The histopathologic examination revealed no significant changes in the hearts or lungs of the test animals. The spleens showed 2+ to 4+ iron, ± iron

however, a 4+ and a 2+ response were found with a guinea pig and a rabbit, respectively, in ¼ hr. after injection. Within 3 hrs. the rabbit also had a 4+ reaction. Other experiments with this same compound confirmed this finding that for a given dosage level fewer Heinz bodies were found in the rabbit than in the guinea pig.

At a dosage level of 50 mgm./kgm., acetylphenylhydrazine by intraperitoneal injection was found to produce 4+ Heinz bodies in mice within ½ hour.

In vitro action of phenylhydrazine was found on mixing whole mouse blood with 7:3 Locke's solution containing 0.5 mgm. per cent of the drug, a few Heinz bodies being seen after 15 minutes, 2+ (35 per cent) after 2 hrs. and 4+ after 5 hrs. When the concentration of phenylhydrazine was increased to 10 mgm. per cent no Heinz bodies could be found, the drug appearing only to attack the erythrocytes and causing them to disintegrate.

Similar experiments with guinea pig blood showed that at 20 mgm. per cent of the drug fewer Heinz bodies were found than when the concentration was 1 mgm. per cent. The chief action in the former case appeared to be the formation of knobby cells and alteration of the cell membrane which took a deep stain with methyl violet. At the lower concentration + (4 per cent) Heinz bodies were found in 1 hr. and 4+ (87 per cent) in 6 hrs. Fig. 4 is a photomicrograph of this wet preparation taken at the 6 hr. interval. Since the cells were moving, only a few of the Heinz bodies are in focus.

It is evident from these experiments that phenylhydrazine and its acetyl derivative are very effective in forming Heinz bodies, and changes in the erythrocytes of the mouse and other species can be detected within a few minutes under suitable conditions.

Action of other chemical agents. Several other chemical substances were investigated during this study, using white mice as experimental subjects, the preparations being given intraperitoneally in aqueous solution. The use of these compounds was suggested either because of their marked toxicity or their known hemolytic action. In a few cases inorganic oxidizing or reducing agents were tried.

Observations for Heinz bodies were made at intervals, the first usually being taken after ¼ hr. Ordinarily evaluations were repeated at 1–4 hr. intervals during the first day and then 24 hrs. later. However, with the very toxic materials at high dosage levels, some of the animals succumbed in a short time after injection, thus preventing further observations. The results are summarized in table 2, the maximum Heinz body rating and the corresponding time being indicated for each substance used.

It can be seen that in general the organic compounds were much more effective than the inorganic materials in inducing Heinz body formation. However, saponin, a powerful hemolytic agent, was much less effective than aniline. A number of the highly toxic inorganic compounds were almost without effect in producing intraerythrocytic particles although their lethal effects were quite evident. The reducing agents, sodium nitrite and sulfite and hydroxylamine, appeared to be superior to the oxidizing agents tried (chlorate, dichromate and

TABLE 2

Summary of Heinz body response of white mice following intraperitoneal injection of various substances

SUBSTANCE	DOSAGE	NO. OF ANIMALS	MAXIMUM HEINZ BODY EVALUATION	TIME OF OBSERVATION AFTER INJECTION
	mgm./kgm.			*hr.*
Aniline	40	2	2+	¼
			±	1
Aniline	400	1	4+	20
Pyrogallol	200	2	+	¼
			±	3
Saponin	50	1	Neg.	4
Saponin	100	2	±	1
			+	3
2–4 Toluylene diamine	100	2	+	3
			Neg.	3
Cobalt chloride	100	1	Neg.	6
Cobalt sulfate	100	1	Neg.	6
Ferrous sulfate	100	2	Neg.	23
			Neg.	23
Hydroxylamine hydrochloride	12.5	1	2+	1
	100	1	3+	¼
Lead acetate	100	1	±	20
Lead acetate	400	1	±	20
Lead chloride	100	1	+	21
Mercuric chloride	5	1	Neg.	4
Mercuric chloride	10	1	+	4
Mercuric chloride	100	1	Neg.	¼
Potassium chlorate	100	2	+ ?	1
			Neg.	20
Sodium dichromate	100	3	2+	1
			Neg.	3
			Neg.	4
Sodium nitrate	500	2	±	1
			Neg.	3
Sodium nitrite	100	1	±	½
Sodium nitrite	200	2	+	¼
			Neg.	½
Tartar emetic	37.5	1	Neg.	96
	100	1	Neg.	½

nitrate) although ferrous sulfate was without effect It is of interest to note that tartar emetic, though exhibiting marked toxicity at the high dosage levels, showed scarcely any effect on Heinz body formation, in marked contrast to the action of antimony in the form of the hydride By far the greatest action for inorganic materials was exhibited by hydroxylamine which is both a nitrogen compound and a reducing agent

In vitro action of hydroxylamine hydrochloride (1 mgm per cent) was observed with mouse blood, a 2+ rating being found 11 minutes after mixing and a 3+ reaction after one half hour At a lower concentration of hydroxylamine (0 25 mgm per cent) the action was slower, the Heinz body ratings being ± in 15 min , + (10 per cent) after 1 hr , 2+ (38 per cent) after 3 hrs and 4+ after 24 hrs It was evident from these observations that the destructive action on the erythrocytes as well as the number and size of Heinz bodies was dependent on the concentration of the drug

DISCUSSION The mechanism of Heinz body formation and the site of their origin have not been elucidated The wide variety of compounds, some of which are inorganic, capable of inducing intraerythrocytic changes, lends support to the view that Heinz body formation is due to partial destruction of some portion of the red blood cell, as a result of toxic action The view that these bodies are formed in the peripheral circulation is supported by the fact that usually only mature cells and not reticulocytes have Heinz bodies and also by the fact that *in vitro* formation of such particles has been demonstrated Although difficult to show photographically, the rapidity of this formation can easily be shown by visual observation using wet blood preparations The more favorable conditions present in the living tissue are presumably responsible for the reaction *in vivo* usually being much greater than that *in vitro* The persistence of Heinz bodies after a single dose of a substance is of great interest since it points to a relatively long life of some of the erythrocytes, amounting to 30–55 days in the mouse

Although Heinz bodies were present in the majority of the erythrocytes of mice fed sulfanilamide during the 6–7 month test period, no hemolytic anemia was evident This is in marked contrast to man, in which the presence of numerous Heinz bodies would be regarded as serious (3) Furthermore, the ability to form Heinz bodies is apparently not related either to the ability to form methemoglobin or to cause hemolysis For example, saponin, which is regarded as a powerful hemolytic agent, was found to be relatively ineffective in inducing Heinz body formation in mice whereas sulfanilamide was able to produce these particles in nearly all erythrocytes in a short time The most toxic substances appear to be those which are able to cause hemolysis, methemoglobin and Heinz bodies

The production of Heinz bodies by stibine, a volatile hydride, is of interest since for many years it was believed that this phenomenon was almost exclusively confined to nitro compounds of the benzene series Little interest appears to have been shown in studying the action of inorganic compounds in this field

The fact that Heinz bodies were not found in suckling mice, the mother of which exhibited numerous intraerythrocytic particles, suggests some sort of a

protective mechanism in the young. It has been shown (14) that in pregnant rabbits treated with sulfanilamide this drug can pass from the maternal to the fetal circulation. Likewise transmission across the placental barrier has been demonstrated for humans (15). Moreover, transmission of this same drug through the milk of nursing mothers has been established (16). Consequently it was expected that the young mice would have Heinz bodies. However, the experiments on implantation and intraperitoneal injection confirmed the earlier observation and indicated a difference in susceptibility of young and mature mice to this drug.

The difficulty of finding a solution capable of maintaining red blood corpuscles of various species without marked morphologic changes during a supravital examination is well known. For this reason some differences in behavior of the Heinz bodies may be expected for various species. However, the great similarity in morphologic appearance, in their staining characteristics and in their physical and chemical behavior to the particles described earlier by Heinz, makes it quite evident that they should be designated as Heinz bodies.

SUMMARY AND CONCLUSIONS

The formation and properties of intraerythrocytic bodies produced by the action of stibine, phenylhydrazine hydrochloride, sulfanilamide and certain other chemical substances have been studied in experimental animals. Moreover, *in vitro* formation has been observed in mouse blood with phenylhydrazine hydrochloride, with sulfanilamide and with hydroxylamine hydrochloride. With respect to their various staining and morphologic characteristics, these particles have been found to conform to the properties of Heinz bodies.

Improved methods for staining Heinz bodies both in wet preparations and in smears have been worked out. Recognition and enumeration of these particles in their early stages have been facilitated by supravital examination of blood.

The formation of Heinz bodies by certain organic and inorganic compounds is dependent on a number of factors, among which are the chemical nature of the compound, dosage, route of administration and species differences. The Heinz bodies themselves differ in time of appearance, abundance, size and persistence.

Stibine and hydroxylamine hydrochloride are powerful inorganic Heinz body-producing substances while among the organic compounds, phenylhydrazine hydrochloride and acetylphenylhydrazine are very vigorous in their action. Sulfanilamide produces many Heinz bodies in mice and the lack of marked systemic effect, even after a period of months, makes this a useful substance for the study of this phenomenon.

Intraperitoneal injection of a toxic substance is a convenient and rapid method of determining its ability to form Heinz bodies. Because of the ease with which these bodies can be produced in mice, this species is a useful experimental animal. Guinea pigs and rabbits are less serviceable since their erythrocytes are more resistant than those of mice. Hemolytic activity and ability of a substance to form Heinz bodies are not uniformly related; nor is the persistence of these particles related to the maximum number found.

Further experimental work is needed in order to answer many of the remaining questions about the composition, site of origin and fate of Heinz bodies

ACKNOWLEDGMENT We wish to thank Drs S S Spicer, B L Horecker and R C Dunn and Mrs D C Peterson and Mr E C Thompson for their aid in this investigation

REFERENCES

1 WEBSTER, S H Unpublished Mss, 1948 To appear in Blood
2 HEINZ, R Virch Arch f path Anat, 122. 112, 1890
3 MOESCHLIN, S Schweiz med Wchnschr, 70 786 1940
4 MOESCHLIN, S Schweiz med Wchnschr, 71 789, 1941
5 MOESCHLIN, S Folia haemat, 65 345, 1941
6 MOESCHLIN, S, AND HURSCHLER, H Schweiz med Wchnschr, 70 972, 1940
7 HURSCHLER, H Arch f exper Path u Pharmakol, 197 438, 1941
8 JÜRGENS, R, AND SCHÜRER, W Schweiz med Wchnschr, 75 1055, 1945
9 WEBSTER, S H, LILJEGREN, E J, AND ZIMMER, D J Stain Technol, 23 97, 1948
10 WEBSTER, S H, LILJEGREN, E J, AND ZIMMER, D J J Biol Photographic A, 16 99, 1948
11 LOCKE, F S Zentralbl f Physiol, 14 670, 1901
12 FIGGE, F H J Anat Rec, 94 461, 1946
13 WEBSTER, S H, LILJEGREN, E J, AND ZIMMER, D J Unpublished work
14 LEE, H M, ANDERSON, R C, AND CHEN, K K Proc Soc Exp Biol and Med, 38 366, 1938
15 SPEERT, H Bull Johns Hopkins Hosp, 63 337, 1938
16 HEPBURN, J S, PAXSON, N F, AND ROGERS, A N Arch Pediat, 59 413, 1942

THE ANTIFILARIAL ACTION OF CYANINE DYES[1]

I. The Relative Antifilarial Activity of a Series of Cyanine Dyes Against Litomosoides Carinii, in Vitro and in the Cotton Rat

LAWRENCE PETERS, ERNEST BUEDING, ARTHUR D. VALK, Jr., AEME HIGASHI and ARNOLD D. WELCH

Department of Pharmacology, School of Medicine, Western Reserve University, Cleveland, Ohio

Received for publication October 28, 1948

The discovery, during a routine testing program, of the marked antifilarial activity of a large series of cyanine dyes when tested, *in vivo* and *in vitro*, against the filarial worm, *Litomosoides carinii*, of the cotton rat, has been reported in several preliminary publications from this laboratory (1, 2, 3). Unfortunately, this filaricidal effect of the cyanines was found to be highly selective. It was not demonstrable against several other filarial species and, in fact, early clinical trials against *Wuchereria bancrofti* have been unsuccessful. However, the experimental findings accumulated during the course of these studies were believed to be of sufficient fundamental importance to warrant their publication in detail.

In the coordinated program organized by the National Research Council, in 1944, for investigations of the chemotherapy of filariasis, several laboratories concentrated their efforts on the extension of existing knowledge of the antifilarial action of organic derivatives of antimony and arsenic (4, 7, 10, 11). In this laboratory, however, investigations were directed toward non-metallic compounds, in the hope of discovering a new approach to the chemotherapy of this disease.

A. The routine testing of compounds for antifilarial activity. Since the two filarial parasites, *W. bancrofti* and *W. malayi*, which occur in the lymphatic system of infested human beings, have not been found in other animal species, it was necessary to conduct these studies with animals infested with a different, but morphologically related, filarial parasite. The timely report of Culbertson and Rose (4) on the chemotherapeutic action of organic antimonials against the filarial worm, *Litomosoides carinii*, in the Florida cotton rat, led to the selection of this same parasite and animal host for these studies. The occurrence of the worms in the pleural cavity made them readily accessible at autopsy for transfer in an intact condition to nutrient media. The size of the animals (under 200 grams) made them suitable for a large scale exploratory program and much to be preferred, for routine testing, to dogs infested with the heart-worm, *Dirofilaria immitis*. An adequate supply of naturally infested cotton rats was maintained by purchase from the Hegener Research Supply Company of Sarasota, Florida.

The "screening" procedure was based on the principle that each compound should be given in an amount approaching that maximally tolerated by the animal host. Schedules

[1] The work described in this series of papers was done, in part, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Western Reserve University (August 1, 1944 to October 31, 1945); in part, under a contract between the Office of the Surgeon General, U. S. Army (November 1, 1945 to December 31, 1946); and in part, with the aid of a grant from the U. S. Public Health Service (since January 1, 1947).

involving frequent dosage were considered preferable, since they would favor the maintenance of a concentration of the drug in the tissue fluids bathing the parasites, and thus would increase the possibility of detecting minimal antifilarial activity in a compound, as a lead for further study. After a short period of experimentation with various schedules, it was decided, wherever possible, to administer compounds intraperitoneally every 8 hours for a total of 18 doses. For injection purposes the animals were driven from their individual wire cloth cages into a tin and wire cloth injector tube which allowed handling and injecting with little danger of being bitten.[2] Autopsy was performed 40 hours after the final injection. The adult filariae were removed aseptically from the pleural cavities and placed in petri dishes containing 10 cc. of sterile nutrient medium[3] for observation (the exact composition of the medium has been stated in the footnote), worms from an untreated rat were observed simultaneously. Characteristically, unaffected worms remained motile for at least 2 days when observed at room temperature. When motility was absent at the time of autopsy, and did not appear within 8 hours after the worms were removed from the rats, the filariae were considered dead.

The effect of drug treatment on the microfilariae, either *in vitro* or *in vivo*, was not studied, since it was known and was confirmed again in the course of these studies, that the susceptibility of these larvae to noxious agents may differ quite markedly from that of the parent worms against which therapy was directed. Also, it was felt that routine "screening" of unrelated chemical substances for filaricidal activity *in vitro* would give information more likely to be misleading than helpful, since a great variety of substances were certain to be too toxic for filariae *in vitro*. However, extensive studies of the metabolism of *L. carinii* were conducted simultaneously by one of us (2, 3, 15), and sufficient information was obtained through this approach to facilitate progress materially when leads were obtained from the "screening" tests in animals. Under such conditions studies *in vitro* became of great importance.

B. The detection of antifilarial properties in the cyanine dyes

Among the many compounds studied, none was found to possess appreciable activity until a member of the group of compounds known as cyanine dyes was tested. This compound, (1-amyl-2,5-dimethyl-3-pyrrole)(1,6-dimethyl-2-quinoline) dimethincyanine chloride (Chemotherapy Center #348), whose structural formula is shown in table I, was completely curative in the maximally tolerated doses used. On further study it became evident that a very high degree of activity was present, since the intraperitoneal injection of 0.1 mgm./kgm., at 8 hour intervals for 18 doses, regularly killed all filarial worms in all treated animals. Though delayed fatalities occasionally occurred when individual doses of 1.8 mgm./kgm. were administered according to above treatment schedules, individual doses of 1.25 mgm./kgm. on this schedule were well tolerated. These findings indicated that the absolute margin of safety was remarkably high.[4]

[2] This apparatus was designed by J. T. Litchfield, Jr., who at the time was working with R. N. Bieter, H. N. Wright and their associates on a similar project involving other members of this series of compounds (8, 9).

[3] The medium consisted of 1 part sterile horse serum, generously supplied by Sharp and Dohme, Inc., and 3 parts of a buffered glucose salt solution which permitted optimal metabolic activity and motility, and had the following composition: 0.137 *M* NaCl, 0.0027 *M* KCl, 0.0003 *M* $CaCl_2$, 0.001 *M* $MgCl_2$, 0.06 *M* sodium phosphate buffer (pH 7.6), 0.02 *M* glucose.

[4] The term "absolute margin of safety" is used to signify a relation that might be expressed by the ratio $\frac{LD_5}{CD_{95}}$, or $\frac{LD_{0-10}}{CD_{90-100}}$, if indications are to be given of the occasional death of an animal on a dose that is usually non-lethal and the occasional failure of a dose to

With the known antifilarial compounds of antimony, such as 'Neostibosan' and 'Anthiomaline', it was found impossible to kill all worms in any single animal even at maximally tolerated doses on similar treatment schedules.

Quantitative studies of the effect of #348 on the number of microfilariae present in the peripheral blood were not made for reasons already given. However, qualititative examination of single drops of peripheral blood in cover glass preparations revealed no gross differences in the degree of microfilaremia present before, during, and at the completion of the period of treatment. Later, quantitative studies with another cyanine dye (#863) (table IV) revealed that a slow decrease in the microfilaria count did occur over a period of some months. This was believed to be due to the spontaneous death of the embryos and their failure to be replaced because of the much earlier death of the adult worms during the period of therapy (16).

C. THE ANTIFILARIAL ACTIVITY OF CYANINE DYE #348 IN VITRO. When filariae from untreated rats were placed in petri dishes containing 10 cc. of the nutrient medium referred to previously, in the presence of various concentrations of #348, motility was lost within one hour at a concentration of 1:100,000. At a concentration of 1:1,000,000, motility was reduced within 2 hours, and up to 50 per cent of the worms were rendered immobile within 7 hours; in the case of the remaining 50 per cent, however, some motility was present after 72 hours, when the motility of control worms began to decrease. At a concentration of 1:10,000,000, no effect on the worms was detectable. It is of interest that the lowest dose of #348, which consistently cured rats following repeated injections was 0.1 mgm./kgm., since, on the basis of rapid and equal distribution throughout the tissues and tissue fluids, this amount of compound could result in a maximal concentration not exceeding 1 part per 10,000,000, prior to the occurrence of degradation or excretion. This point will be discussed further in a subsequent communication (16).

On the basis of the concurrent studies of the metabolism of *L. carinii* by Bueding (2, 3, 15), it seemed of greater and more fundamental importance to determine the effect of #348 on the specific metabolic behavior of the parasite, than on a general phenomenon such as motility. Thus, it was found that the cyanines produced a marked inhibition of the oxygen consumption of the adult filariae. The oxidative metabolism of the worm was inhibited by the concentrations of #348 ranging from 1:25,000,000 to 1:6,000,000. This decrease in respiration was associated with a compensatory increase in glycolysis and a decrease in glycogen synthesis. Under anaerobic conditions, on the other hand, no effect on glycolysis was observed. Only with concentrations 1,000 to 2,000

cure that is usually curative. The statistically valid margin of safety or therapeutic index, $\frac{LD_{50}}{CD_{50}}$, though an experimentally much more reproducible value, often gives an impression of innocuousness that is not justified by the facts. Although many cyanines, administered either orally or intraperitoneally, have wide "absolute margins of safety", they are by no means as innocuous as the ratio $\frac{LD_{50}}{CD_{50}}$ would indicate.

times greater was the oxygen consumption of mammalian tissue slices or homogenates affected by these compounds, a fact which is reflected in the high margin of safety in the chemotherapeutic tests described earlier. Furthermore, worms removed from cotton rats treated with subcurative doses of #348 showed markedly depressed respiratory activity and an increase in aerobic glycolysis, as compared to worms removed from untreated rats. It seemed probable, therefore, that these drugs exert their chemotherapeutic effect through the inhibition of one or more enzyme systems concerned with oxidative metabolism. The metabolism of the microfilariae obtained from pleural washings was unaffected by the drug, a circumstance that may be attributed to a difference in the metabolic characteristics of the larvae from those of the adult parasites (2, 3, 15). This observation is in agreement with the finding that the drug caused no prompt reduction in the microfilaremia of the cotton rat.

D. COMPARATIVE STUDIES OF THE ANTIFILARIAL ACTIVITY OF CYANINE DYES. It seemed highly desirable to extend these studies to other cyanine dyes because of the high degree of curative activity, the high margin of safety, and the specific antimetabolic effect of the one compound tested. Furthermore, Bieter, Wright and their associates had simultaneously disclosed antifilarial properties in a related series of styrylquinoline dyes (8, 9).

Fortunately for the chemotherapeutic program, a large number of these compounds had been synthesized by Dr. L. G. S. Brooker and his associates of the Eastman Kodak Company for other purposes (12, 13). Samples of these were generously submitted to the Committee on Medical Research of the National Research Council through Parke, Davis and Company, for study in this laboratory.[5]

Two procedures were used in screening these compounds for antifilarial activity against *L. carinii*. The first of these involved a comparison of the ability of the various cyanines to inhibit the oxygen uptake of the parasites. The second consisted of an assay, in cotton rats, of the chemotherapeutic and toxic properties of each compound.

1. *Assay, in vitro, of the antifilarial activity of cyanine dyes.* Adult filariae (15 to 20 mgm.) were transferred to small Warburg vessels (volume, 4 to 5 cc.) which contained 0.7 cc. of buffered glucose-salt solution.[3] A small roll of filter paper, soaked with 0.1 cc. of 40 per cent KOH was placed in the center cup of each vessel. The oxygen uptake of the worms was measured over a period of 3½ hours at 38°C. in an atmosphere of air, in the conventional Warburg apparatus. During the initial period of 30 minutes, a slight increase in the respiration of the worms frequently occurred. After this time, the rate of oxygen uptake of the filariae remained constant over a period of at least three to four hours. The control respiration was recorded for the next 90 minutes, i.e., 30 to 120 minutes after the beginning of the experiment. Following this contol period, 0.1 cc. of the glucose medium containing a cyanine dye was tipped from the side-arm into the main compartment of the vessel and the respiration was recorded for an additional 90 minutes. Addition of 0.1 cc. of the same

[5] From the beginning of the study of this group of compounds, we have enjoyed the finest cooperation from Dr. Brooker and from Parke, Davis Laboratories. Further, this investigation has been facilitated in innumerable ways by Dr. Lucille Farquhar, technical aide of the National Research Council, who coordinated studies in this and related fields.

medium containing no cyanine dye did not affect the rate of oxygen uptake of the worms. All solutions of compounds to be tested were prepared immediately before the experiment. The antifilarial activity of each compound, *in vitro*, designated in the tables as the "*in vitro* index", was established in the following manner. The molar concentration of compound #348, which was used as a standard of reference, required to inhibit the respiration of the worms by 50 per cent (usually 2.6×10^{-7} *M* or 1:10,000,000) was divided by the molar concentration producing a similar decrease in respiration in the case of the compound under assay.

2. *Assay, in vivo, of the antifilarial action of cyanines.* Further studies on the antifilarial action of #348 in cotton rats indicated that cures could be produced when dosage intervals of much greater than 8 hours were used. The minimal curative dose, when the drug was administered intraperitoneally at 8-hour intervals for 6 days, was 0.1 mgm./kgm.; a total dose of 1.8 mgm./kgm. When one injection was given daily for 5 days, an increase in the individual dose to only 0.2 mgm./kgm. was required; a total dose of 1.0 mgm./kgm. Furthermore, when the interval between the last dose and the time of autopsy was extended to 4 days, 0.3 mgm./kgm. daily for 3 days was required. When a single dose was given, followed by a period of one week, prior to autopsy, it was found that 1.35 mgm./kgm. would effect a cure. Later, the studies with other cyanines yielded similar results.

Accordingly, in "screening" various related compounds for their chemotherapeutic activity against *L. carinii* in the cotton rat, a dosage schedule was selected which involved one injection daily for 5 days. Autopsies, involving removal of worms from the pleural cavity for the purpose of observing the effect of therapy on motility, as described earlier, were performed 48 hours after the last dose. The following dose levels, expressed as the amount administered daily in mgm./kgm. of body weight, were used: 0.05, 0.10, 0.15, 0.20, 0.30, 0.40, 0.80, 1.6 or 2.0, 3.0, 4.0, 6.0, 8.0, 12.0, 16.0, etc. It was not necessary to use all these dose levels for each compound, and the data for any one compound were not obtained in a single test. Rather, an orientation test was performed first by treating several infested rats at one of the lower dose levels, and several uninfested rats at one of the higher dose levels. On the basis of the results obtained, higher or lower doses were then used in further experiments on additional rats to determine minimally curative and maximally tolerated doses. In this preliminary "screening" program, designed to separate compounds of high chemotherapeutic activity worthy of more extensive investigation, from those of low activity, only three animals were used per dose level of drug. The large number of compounds to be studied, the high cost of the infected animals, and the special handling which these separately housed animals required during maintenance, and especially during injection, made it necessary to set such a low figure for the number of animals to be used per drug. The values determined for each compound were: first, the minimum dose required to produce death of all worms in the three rats of a dosage group (minimum curative dose); and, second, the maximum dose allowing survival of all three rats in a dosage group (maximum tolerated dose). The margin of safety, or therapeutic index, was calculated by dividing the second value by the first. Such a therapeutic index was selected in preference to one involving 50 per cent end points, not only because of the small number of animals used, but also, as has been mentioned previously, because values representing complete curative responses, and complete survival, seemed more comparable to a clinical situation. Because both the toxicity and curative activity of these compounds increase rather slowly with increasing dosage, the statistical therapeutic index, based on 50 per cent responses, would have yielded figures of considerably higher magnitude.

In the data recorded in the following tables, the toxicity tests were performed with uninfested cotton rats of a single strain[6]. Earlier toxicity tests with a few cyanines, including #348, were performed with uninfested rats obtained from the same natural habitat as the infected ones. These last-mentioned rats were considerably more resistant to the lethal effects of the cyanines, than were those of the laboratory strain; hence, con-

[6] Obtained from Tumblebrook Farms, Brant Lake, New York.

siderably higher therapeutic indices resulted from their use Since our interest was in a comparison of the relative toxicity of these compounds so far as these lower animals were concerned, rather than in absolute values, we did not revert to the use of Florida rats for toxicity studies when this discrepancy became apparent Rather, we repeated the earlier toxicity tests, on the daily injection schedules, with the laboratory strain of rats which were used thereafter

In the case of some of the compounds presented in the tables, the "*in vitro* index" alone was determined In almost all such cases the amount of sample available was insufficient to permit studies *in vivo* The small samples sufficed for the metabolic studies, and yielded additional information with regard to the relation of chemical structure to antifilarial activity In a few other cases assays were performed only *in vitro* with compounds, other than cyanines, which were well known from work in this and in other laboratories to be devoid of any chemotherapeutic effect *in vivo* In this way it was possible to determine the presence or absence of a low degree of antifilarial activity, the existence of which was suggested by a remote chemical similarity to the cyanines The results of the "screening" tests are presented in tables I to X

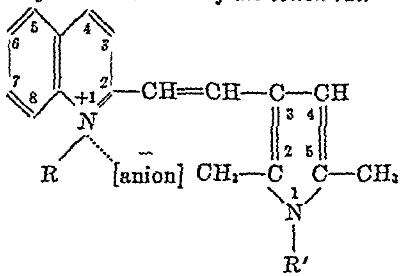
E THE RELATION OF THE CHEMICAL STRUCTURE OF THE CYANINE DYES TO THEIR ANTIFILARIAL ACTIVITY AGAINST L CARINII It has been postulated earlier that the cyanines exert their chemotherapeutic effect through the inhibition of one or more enzyme systems concerned with the oxidative metabolism of the parasite Further evidence for this, presented in the tables, is shown by the fact that every compound found to be active *in vivo* also inhibited filarial respiration *in vitro* The fact that the reverse correlation did not exist, in other words, all compounds active *in vitro* were not active *in vivo*, is not a refutation of this postulate In the complex animal organism many factors, such as rate and extent of excretion, and metabolic alteration, as well as unfavorable distribution, indubitably influenced the extent to which the inherent antifilarial activity of various compounds was able to become manifest Thus, a discussion of the relation between chemical structure and the inherent antifilarial activity of these compounds would be more valid when based on observations made *in vitro*, in the absence of the modifying factors imposed by an animal host Such a discussion is presented below, with supplementary remarks on the relation between chemical structure and "net" antifilarial activity, i e, activity *in vivo* Accordingly, the term "activity", as used in the following discussion, will refer to antifilarial activity *in vitro*, unless otherwise stated The various related members of the cyanine series made available to us were prepared largely for purposes other than the chemotherapeutic program (12, 13) In a number of cases, however, the group led by Dr Brooker kindly prepared new compounds, the structure of which was suggested by the antifilarial studies

As shown in the tables, many different types of cyanines were found to possess antifilarial activity, and the synthesis of numerous derivatives of every structural type was impractical, at least until some knowledge was forthcoming in regard to the effectiveness of the cyanines against *W bancrofti* in man

1 *(3 Pyrrole)(2 quinoline) dimethincyanines (table I)* The most complete study of the effect of various substituent groups on antifilarial activity, both *in vitro* and *in vivo*, was made with this group of compounds which included #348, the cyanine originally found to be active against *L carinii* In compounds

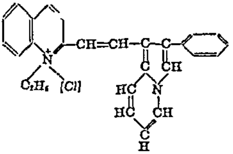
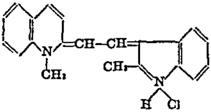
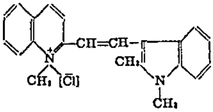
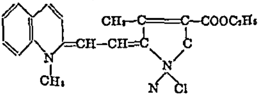
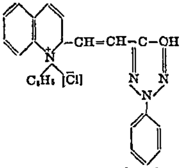
TABLE I

The relative antifilarial activity of a number of (3-pyrrole)(2-quinoline) dimethincyanines against L. carinii of the cotton rat.



CHEMOTHERAPY CENTER NO.	SUBSTITUENT GROUP: 1 — R	SUBSTITUENT GROUP: 1 — anion	R′	6	7	8	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
							mgm./kgm.	mgm./kgm.		
348	CH_3	Cl	C_5H_{11}	CH_3			0.2	2.0	1.0	*
804	CH_3	Cl	CH_3	CH_3	—	—	>16.0	16.0	<1.0	0.5
711	CH_3	Cl	C_2H_5	CH_3	—	—	8.0	8.0	1.0	1.5
999	CH_3	Cl	C_3H_7(n)	CH_3	—	—	2.0	4.0	2.0	1.5
1093	CH_3	Cl	C_3H_7(iso)	CH_3	—	—	1.6	4.0	2.5	1.0
998	CH_3	Cl	C_4H_9	CH_3	—	—	2.0	2.0	1.0	1.25
348	CH_3	Cl	C_5H_{11}	CH_3	—	—	0.2	2.0	10.0	1.0
802	CH_3	Cl	C_7H_{15}	CH_3	—	—	0.2	1.0	5.0	0.4
1092	CH_3	Cl	$C_{10}H_{21}$	CH_3	—	—	0.2	1.0	5.0	0.5
712	CH_3	Cl	$C_{12}H_{25}$	CH_3	—	—	0.4	<4.0	<10.0	0.1
997	CH_3	Cl	C_6H_{11}(cyclo)	CH_3	—	—	0.4	2.0	5.0	1.5
805	CH_3	Cl	$C_{16}H_{33}$	CH_3	—	—	—	—	—	0.002
799	CH_3	$CH_3C_6H_4SO_3$	C_6H_5	CH_3	—	—	—	—	—	0.8
713	CH_3	Cl	C_6H_5	OCH_3	—	—	0.8	8.0	10.0	0.25
818	CH_3	Cl	C_6H_5	C_6H_5	—	—	—	—	—	0.30
714	CH_3	Cl	$C_6H_4OC_2H_5$	CH_3	—	—	0.3	<3.0	<10.0	0.6
715	CH_3	Cl	C_6H_5	$N(CH_3)_2$	—	—	1.6	6.0	4.0	0.7
800	CH_3	Cl	C_6H_5	—	—	—	>0.8	<3.0	<4.0	0.7
808	CH_3	Cl	C_6H_5	—	—	CH_3	—	—	—	1.0
803	CH_3	Cl	C_6H_5	—	—	OCH_3	0.8	2.0	2.5	0.7
819	CH_3	Cl	C_6H_5	—	—	C_6H_5	—	—	—	0.25
807	CH_3	Cl	C_6H_4Cl	CH_3	—	—	0.8	<4.0	<5.0	0.6
815	CH_3	Cl	C_6H_5	$NH\cdot CO\cdot C_5H_{11}$	—	—	>2.0	2.0	<1.0	0.05
811	CH_3	Cl	C_6H_5	Cl	—	—	>6.0	7.5	1.0 or <1.0	0.4
812	CH_3	Cl	C_6H_5	—	—	Cl	>4.0	4.0	<1.0	1.0
816	CH_3	Cl	C_6H_5	—	Cl	—	1.6	4.0	2.5	0.3
962	CH_3	Cl	C_5H_{11}	—	Cl	—	>1.6	4.0	<2.5	0.7
814	CH_3	Cl	C_6H_5	—	CH_3	—	>4.0	4.0	<1.0	1.0
817	CH_3	Cl	C_6H_5	C_5H_{11}(tert)	—	—	>1.6	<1.6	<1.0	0.4
959	H	Cl	C_6H_5	CH_3	—	—	>128.0	128.0	<1.0	0
963	C_2H_5	Cl	C_5H_{11}	CH_3	—	—	0.3	4.0	13.0	1.5
349	C_2H_4OH	Cl	C_5H_{11}	CH_3	—	—	3.2	8.0	2.5	0.5
964	C_3H_7	Cl	C_5H_{11}	CH_3	—	—	0.15	2.0	13.0	0.8
965	C_5H_{11}	Cl	C_5H_{11}	CH_3	—	—	0.15	2.0	13.0	0.5
943	CH_3	Cl	C_5H_{11}	—	—	—	0.40	4.0	10.0	1.0
967	CH_3	Cl	C_5H_{11}	OCH_3	—	—	0.40	6.0	15.0	1.0
806	CH_3	Cl	$CH_2CH_2OCH_3$	CH_3	—	—	>2.0	2.0	<1.0	0.5

TABLE I—*Continued*

CHEMOTHERAPY CENTER NO	SUBSTITUENT GROUP: 1 — R	SUBSTITUENT GROUP: 1 — anion	SUBSTITUENT GROUP: R′	SUBSTITUENT GROUP: 6	SUBSTITUENT GROUP: 7	SUBSTITUENT GROUP: 8	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
							mgm / kgm	*mgm / kgm*		
801	C_2H_5	Cl	C_6H_5	CH_3	—	—	—	—	—	0 8
809	C_7H_{15}	Cl	C_6H_5	CH_3	—	—	0 8	2 0	2 5	0 6
810	$C_{11}H_{23}$	Cl	C_6H_5	CH_3	—	—	>4 0	4 0	<1 0	>0 1
813	$CH_2CH_2OC_2H_5$	Cl	C_6H_5	CH_3	—	—	0 8	2 0	2 5	0 6
820	C_6H_5	Cl	C_6H_5	—	—	—	1 6	3 2	2 0	0 8
1276	CH_3	Cl	$CH_2C_6H_5$	CH_3	—	—	0 8	2 0	2 5	1 0
1275							—	—	—	0 7
797							>16 0	16 0	<1 0	0 005
798							0 8	8 0	10	0 5
821	 a (2 pyrrole) (2-quinoline) dimethincyanine						—	—	—	<0 02
1278	 a (3 triazole) (2-quinoline) dimethincyanine						>16 0	16 0	<1 0	0 05

* Standard of reference (1 0)

having a methyl group in position −1 or −6 of the quinoline ring, the highest degree of activity was observed when an ethyl (#711), propyl (#999), or a cyclohexyl (#997) radical was attached to the pyrrole-N. A somewhat lower degree of activity was present when a methyl (#804), butyl (#998), or amyl (#348) radical was the substituent. A further lengthening of the side-chain produced a progressive decrease in activity (#802, #1092, #712, #805). Activity *in vivo*, on the other hand, was poor when the alkyl substituent on the pyrrole-N was short, and increased gradually to a maximum at C_5 through C_{10}, beyond which it could not be tested with accuracy due to the low degree of solubility, and of absorbability from the site of injection.

It is regrettable that the series of analogues discussed in the above paragraph, i.e., those having methyl groups in position -1 and -6 of the quinoline ring, with various substituents on the pyrrole-N, did not include the compound in which a phenyl group was the variant. The comparative effect of a phenyl group versus *one* alkyl radical, namely, the amyl, can be seen, however, in *other* compounds of the (3-pyrrole)(2-quinoline) series of table I, where substituents in other positions were the same. Thus, in each of the following four pairs of compounds the amyl radical conferred greater activity on the compound than did the phenyl: #713 and #967; #800 and #943, #962 and #816; #801 and #963. For the first two pairs of compounds this was also true *in vivo*, while for the third pair the phenyl compound was more active in cotton rats; a comparison was not made with the fourth pair.

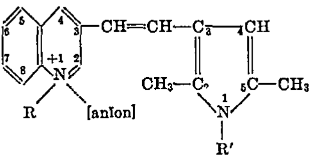
Data are also available in table I for a study of the effect on antifilarial activity of various substituents at the quaternary quinoline-N. With an amyl group on the pyrrole-N, replacement of a methyl group (#348) by an ethyl group (#963) at the quinoline-N produced an increase in activity, while further lengthening of the substituent alkyl chain led to decreased activity (#964 and #965). In this case, the changes in activity *in vivo*, though not marked, followed the reverse order. Replacement of the ethyl group of #963 by a β-hydroxyethyl group (#349) produced a marked decrease in activity both *in vitro* and *in vivo*. The deleterious effect of a long alkyl chain on the quinoline-N was also seen in comparing the relative activities of compounds whose pyrrole-N carried a phenyl radical, though it was outstanding only in going from a heptyl to an undecyl substituent (#801, #809, #810).

The effect of substituents at other positions than the nitrogens can be seen in various compounds with the common feature of a methyl radical on the quinoline-N and a phenyl on the pyrrole-N. When #800, which had no additional substituent, was altered by exchanging the hydrogen at C-8 for a methyl group (#808), or a chlorine atom (#812), a slight increase in activity resulted. When the methyl group was in position-6 (#714), instead of in position-8 (#808), a reduction in activity occurred. Similarly, decreased activity was observed with a chlorine atom in position-6 (#811) or -7 (#816), instead of in position-8 (#812) of the quinoline ring. A methoxy group in position-8 (#803) had no effect on activity, but a considerable decrease in activity occurred with a methoxy group in position-6 (#713). A phenyl group in position-6 (#819 or -8 (#818) resulted

in a decrease of activity of the same magnitude Thus, compounds possessing —Cl (#812), —CH_3 (#808), or —OCH_3 (#803) in position 8 of the quinoline ring appeared to possess higher activity than those in which a similar substituent was introduced in position 6 or -7 On the other hand, when no substituent was present in the 6, -7 or -8 position of the quinoline ring, as in #943, the antifilarial activity *in vitro*, remained equal to that of #348, which carried a methyl group at position-6 In the cotton rat the former compound was less active than the latter, but a corresponding decrease in toxicity permitted the therapeutic index to remain unchanged

TABLE II

The relative antifilarial activity of a number of (3 pyrrole)(4 quinoline) dimethincyanines against L carinii of the cotton rat



CHEMO THERAPY CENTER NO	SUBSTITUENT GROUP 1 R	anion	R	6	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERA PEUTIC INDEX	IN VITRO INDEX
					mgm /kgm	*mgm /kgm*		
823	CH_3	Cl	C_6H_5	—	—	—	—	0 3
824	CH_3	Cl	C_6H_5	OCH_3	1 6	24 0	15 0	0 25
955	CH_3	Cl	C_5H_{11}	—	0 8	2 0	2 5	0 8
1095	C_2H_5	Cl	C_5H_{11}	—	0 4	2 0	5 0	1 0
1096	C_3H_7	Cl	C_5H_{11}	—	0 2	1 0	5 0	0 7
1097	CH_2—CH OH	Br	C_5H_{11}	—	>1 6	16 0	<10 0	0 3
1277	C_2H_5	Cl	$C_{10}H_{21}$	—	0 8	4 0	5 0	0 15

2 (*3 Pyrrole*)(*4-quinoline*) *dimethincyanines* (*table II*) As in the first group of compounds, greater activity was observed here when an amyl radical was attached to the pyrrole N than when a phenyl was present (#955 and #823) Alteration of #823, by insertion of a methoxy group in position 6 of the quinoline moiety, did not significantly alter activity An increase in the length of the alkyl radical on the quinoline N augmented activity when the new substituent was an ethyl radical (#1095), but decreased it slightly with a propyl group (#1096) As in the previous group of cyanines, a β hydroxyethyl group very significantly decreased activity The detrimental effect, on activity, of an excessively long alkyl chain on the pyrrole N is demonstrated again by comparison of the "*in vitro* index" of #1095 (amyl) and #1277 (decyl)

In regard to the effect of moving the attachment of the cyanine bridge from

TABLE III

The relative antifilarial activity of a number of cyanines of the diquinoline type against L. carinii of the cotton rat

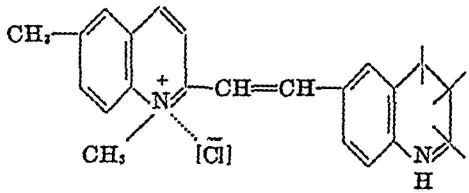
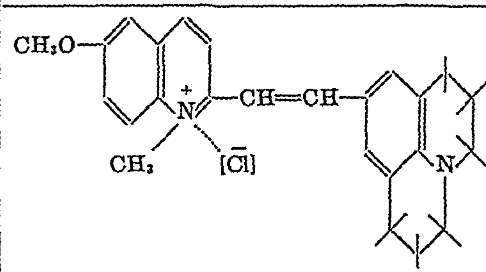
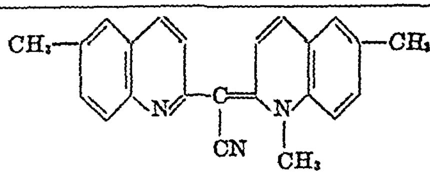
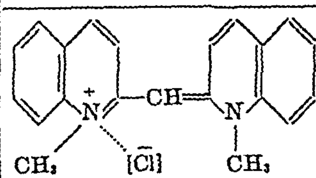
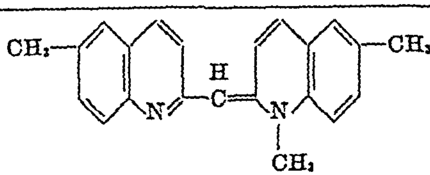
CHEMOTHERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
	Dimethincyanines				
		mgm./kgm.	*mgm./kgm.*		
834		>3.2	6.0	<2.0	2.0
837		>4.0	<8.0	<2.0	1.0
	Monomethincyanines				
853		—	—	—	0
856		—	—	—	0.3
854		—	—	—	0.004

TABLE III—*Continued*

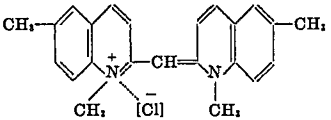
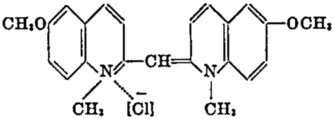
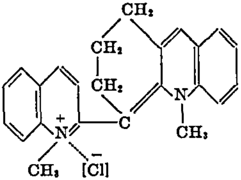
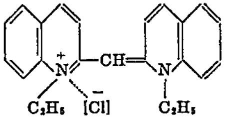
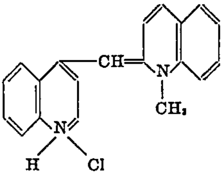
CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
	Monomethincyanines—*Continued*				
		mgm/kgm	*mgm/kgm*		
858		>4 0	4 0	<1 0	0 7
865		—	—	—	0 5
892		3 2	12 0	4 0	1 0
947		>2 4	<4 0	<2 0	1 0
855		—	—	—	0

TABLE III—*Continued*

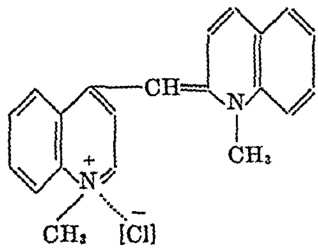
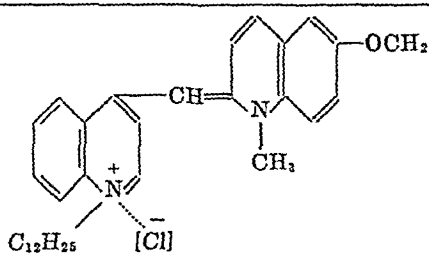
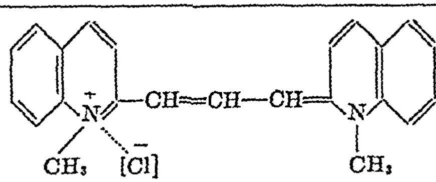
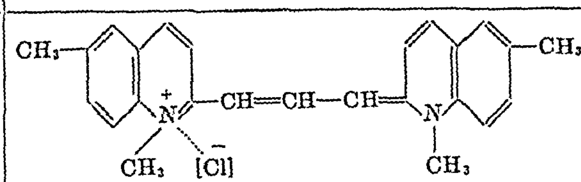
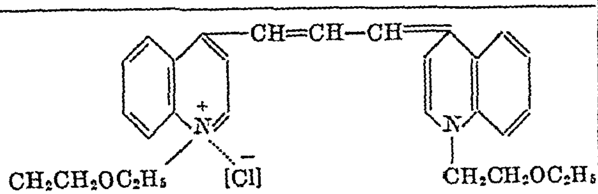
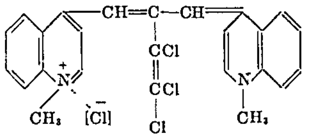
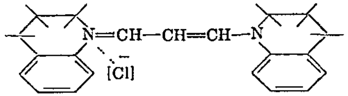
CHEMOTHERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
	Monomethincyanines—*Continued*				
		mgm./kgm.	*mgm./kgm.*		
857		>4.0	>6.0	<1.5	0.5
866		0.8	2.0	2.5	0.1
	Trimethincyanines				
871		0.15	2.0	13.0	0.3
869		>0.4	<0.8	<2.0	0.25
882		4.0	<6.0	<1.5	0.15

TABLE III—*Concluded*

CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
	Trimethincyanines—*Concluded*				
		mgm / kgm.	*mgm / kgm*		
884		—	—	—	0 25
1000		—	—	—	0.15

position-2 to position-4 of the quinoline ring, no difference was observed in the activity of #713 (table I) and #824 (table II). The decrease in the activity of #824 *in vivo* was compensated for by its lower degree of toxicity, so that a slightly higher therapeutic index resulted. In several other instances where the cyanine bridge was moved to the new position, a decrease in activity *in vitro* was observed (#800 and #943 of table I versus #823 and #955, respectively, of table II). Numbers 943 and 955 both were tested chemotherapeutically and a similar difference in their potency was observed *in vivo*.

3. *Diquinoline cyanines* (*table III*). One striking factor involved in the relation of structure to pharmacological action of the cyanines was demonstrated in this group; this pertains to the nature of the connecting bridge between the two nitrogens. The three compounds in which both nitrogens were tertiary (#853, #854 and #855) showed no appreciable activity. The conversion of one nitrogen to the quaternary form resulted in the establishment of a resonating system of alternating double and single bonds in the chain connecting the two nitrogens. This was associated with a marked degree of antifilarial activity that was sustained despite many minor modifications in structure (table III).

The effect of a shift in the cyanine bridge from the 2-2[1] position to the 2-4[1] position, as shown in #856 and #857, was a moderate increase in activity.

No difference in antifilarial activity *in vitro* was present between the monomethine cyanine, #856, and the corresponding trimethine derivative. A similar comparison of #858 and #869, however, showed the monomethine compound to be considerably more active, since the addition of methyl groups in the 6-positions of the two quinoline rings increased appreciably the activity of the monomethine, but not that of the trimethine compound. A comparison of this sort was also made with two compounds of the dipyrrole type (#1113, #1114);

here the monomethine compound was very much more active than its trimethine analogue.

Few compounds were available in which alkyl radicals other than methyl were attached to the nitrogens in this series. The change from a methyl to an ethyl substituent was studied in the case of #856 and #947. It resulted in a threefold increase in activity.

The two dimethine compounds in this series were not analogous to any of the dimethincyanines of the pyrrole-quinoline type (tables I and II), hence no comparisons of activity could be made in this case.

A number of the compounds in this series showed a high degree of antifilarial activity *in vitro*. In fact, one of these, #834, had an "*in vitro* index" of 2.0, and, though several other cyanines equaled this potency, none of them exceeded it. Despite this, #834 did not have a high degree of antifilarial activity in the cotton rat. In fact, the only diquinoline compound which was highly active from the chemotherapeutic standpoint was #871. The "*in vitro* index" of this compound was not outstanding (0.3); hence this series affords a good example of the fact that the animal host may influence profoundly the antifilarial activity of the cyanines. It is interesting also that Bieter, Wright and their associates (8, 9) have reported a 2-2^1 trimethincyanine of the diquinoline type to have a high degree of antifilarial activity and a high therapeutic index in infested cotton rats; both nitrogens in this compound carried β-ethoxyethyl groups.

4. *Two compounds which deserve special mention* are #863 and #835 (table IV), whose structures are unique in that the second heterocyclic ring in each case was not present in any other of the compounds studied; each had an "*in vitro* index" of 2.0. In both cases the chemotherapeutic activity in infested cotton rats was not of a maximal order, but because their toxicity also was less than that of many other cyanines, the high therapeutic index which resulted made them members of the group of compounds selected for further study.

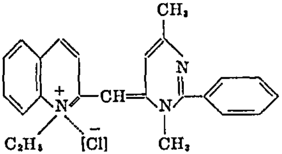
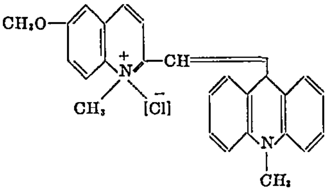
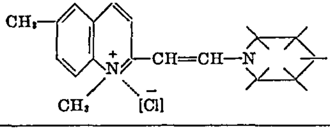
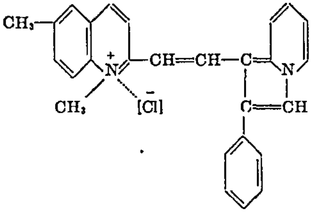
5. *The pyrrole-pyridine cyanines* depicted in table V were not outstanding in their activity, but they merit attention because of the deleterious effect produced by replacement of an amyl group on the pyrrole-N by a phenyl radical. This decrease was of a much greater magnitude than that resulting from a similar change of substituents in the pyrrole-quinoline series (table I). It may be pointed out also that the pyrrole-pyridine compound, #957, was half as active as its corresponding pyrrole-quinoline analogue (#943, table I).

6. *A number of pyrrole-benzimidazole and pyrrole-benzothiazole cyanines* were available for study (tables VI and VIII), and in a few cases "*in vitro* indices" of 1.0 were obtained. However, this high degree of activity did not carry over to the chemotherapeutic assays. A few pyrrole-benzoxazole (table VII) and pyrrole-benzoselenazole (table VIII) compounds were also studied, but none was outstandingly active, either *in vivo* or *in vitro*. In these cyanine types, replacement of an amyl by a phenyl radical was not detrimental to activity as was the case in previous series; in fact, in one case the activity was actually enhanced by such a change (#1091 and #822, table VI).

7. *The styryl-quinoline compounds* listed in table X have been studied ex-

TABLE IV

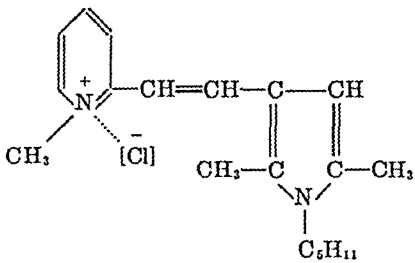
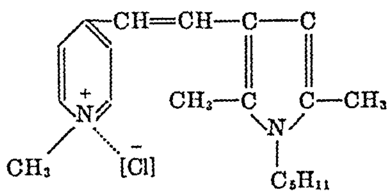
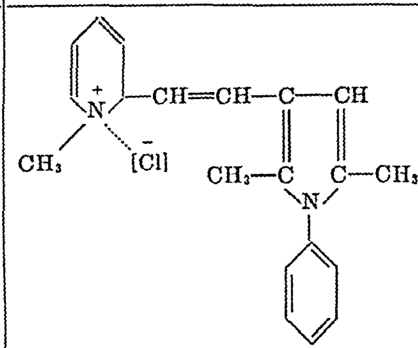
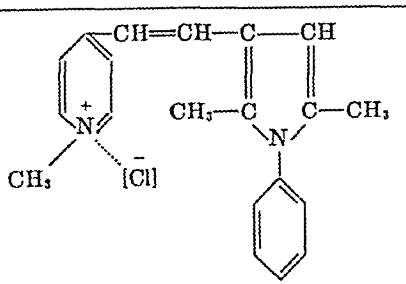
The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a quinoline ring, and the tertiary nitrogen in a heterocyclic ring other than quinoline or pyrrole

CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm / kgm	*mgm / kgm*		
863		0 4	4 0	10 0	2 0
862		3 2	<6 4	<2 0	0 4
831		>8 0	8 0	<1 0	0 25
835		0 4	4 0	10 0	2 0

tensively in regard to chemotherapeutic activity in infested cotton rats by Bieter, Wright and their associates (8, 9). As in the case of the compounds previously discussed, these contain a quaternary and a tertiary nitrogen con-

TABLE V

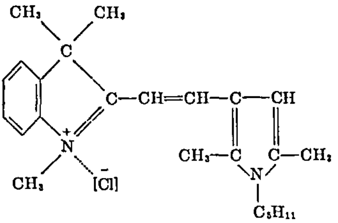
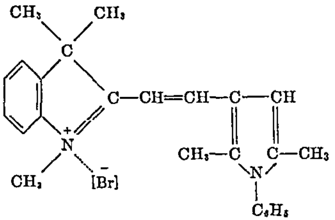
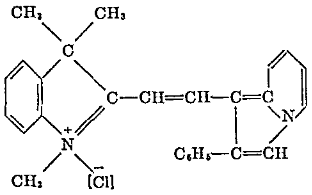
The relative antifilarial activity of several (3-pyrrole)(2-pyridine) and (3-pyrrole)(4-pyridine) dimethincyanines against L. carinii of the cotton rat

CHEMO-THER-APY CENTER NO.	STRUCTURE	MINI-MUM CURA-TIVE DOSE	MAXI-MUM TOLER-ATED DOSE	THERA-PEUTIC INDEX	IN VITRO INDEX
		mgm./kgm.	*mgm./kgm.*		
957		4.0	8.0	2.0	0.5
958		>1.6	<2.0	<1.0	0.5
825		>8.0	<16.0	<1.0	0.025
826		>4.0	8.0	<2.0	0.06

nected by a carbon chain whose bonds are alternately single and double They differ, however, in that the tertiary nitrogen is not a member of the heterocyclic ring, but rather is a para amino nitrogen Compound #350, in which two ethyl

TABLE VI

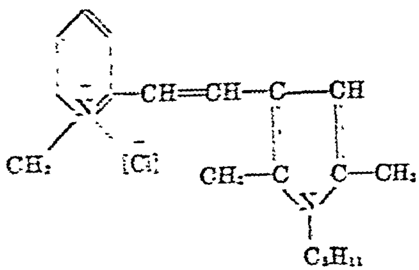
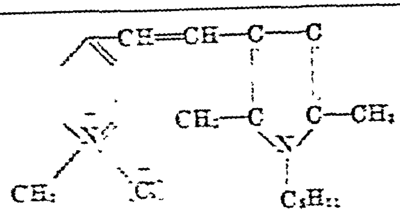
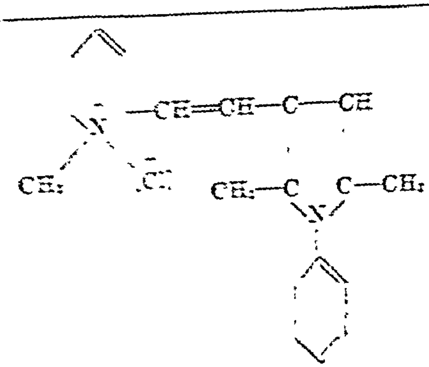
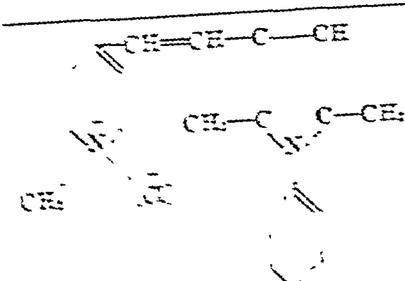
The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a benzimidazole ring

CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm / kgm	*mgm / kgm*		
1091		3 2	<6 4	<2 0	0 4
822		>4 0	12 0	<3 0	1 25
838		0 8	<4 0	<5 0	0 25

groups were attached to the tertiary-N and an ethyl group was the substituent at the quaternary quinoline-N, was outstandingly active *in vitro*, though this high degree of activity was not retained in the cotton rat The remaining

TABLE V

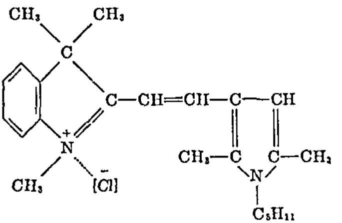
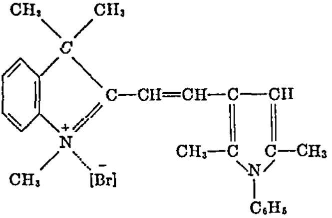
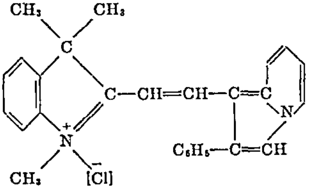
The relative antifilarial activity of several (3-pyrrole)(2-pyridine) and (3-pyrrole)(4-pyridine) dimethinecyanines against L. carinii of the cotton rat

CHEMO-THERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm./kgm.	*mgm./kgm.*		
957		4.0	8.0	2.0	0.5
955		>1.6	<2.0	<1.0	0.5
825		>8.0	<16.0	<1.0	0.025
[illegible]		>4.0	8.0	<2.0	0.06

nected by a carbon chain whose bonds are alternately single and double. They differ, however, in that the tertiary nitrogen is not a member of the heterocyclic ring, but rather is a para-amino nitrogen. Compound #350, in which two ethyl

TABLE VI

The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a benzimidazole ring

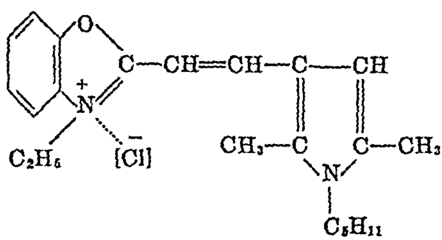
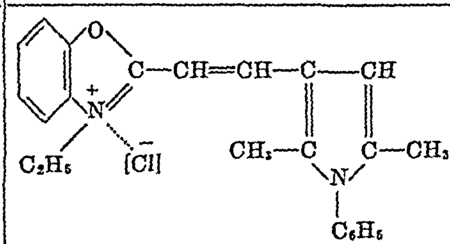
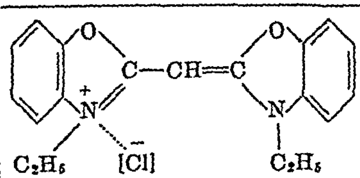
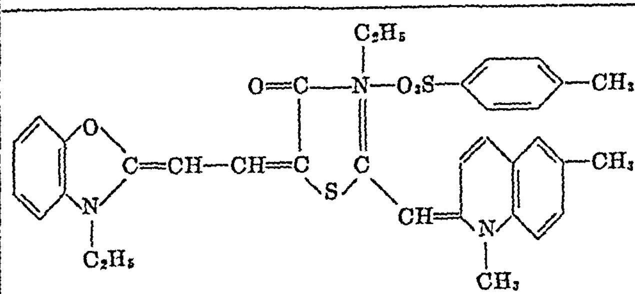
CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm / kgm	mgm / kgm		
1091		3 2	<6.4	<2 0	0.4
822		>4.0	12 0	<3 0	1.25
838		0.8	<4.0	<5 0	0.25

groups were attached to the tertiary-N and an ethyl group was the substituent at the quaternary quinoline-N, was outstandingly active *in vitro*, though this high degree of activity was not retained in the cotton rat. The remaining

compounds of this group all had a methyl, rather than an ethyl substituent on the quinoline-N. Variants of these involved particularly the amino-N, where

TABLE VII

The relative antifilarial activity of a number of cyanines in which the quaternary nitrogen is present in a benzoxazole ring

CHEMOTHERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm./kgm.	mgm./kgm.		
966		>4.0	8.0	<2.0	0.4
829		>1.6	<4.0	<2.5	0.5
K-193		—	—	—	0.25
891		—	—	—	0.2

di-iso-propyl substitution yielded considerably higher activity than di-n-propyl or d-n-butyl substitution, while di-n-amyl substitution yielded a compound of

TABLE VIII

The relative antifilarial activity of a number of cyanine dyes in which the quaternary nitrogen is present in a benzothiazole ring or a benzoselenazole ring

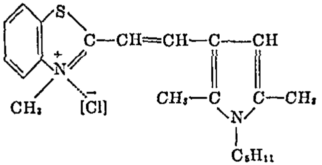
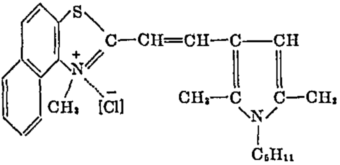
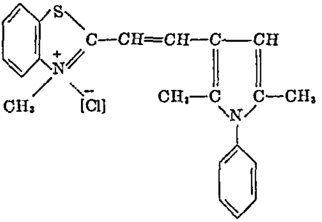
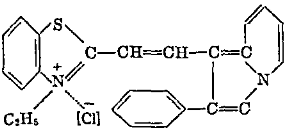
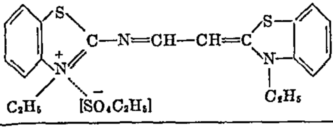
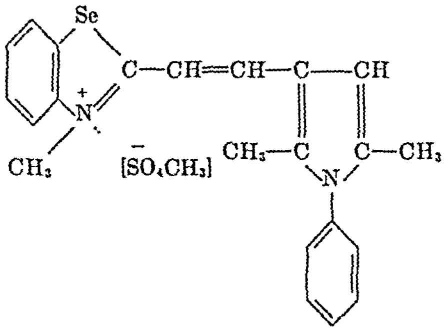
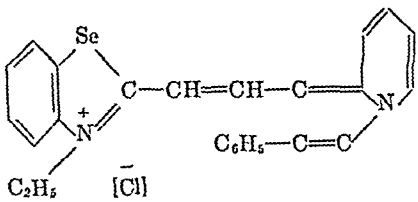
CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm./kgm	*mgm./kgm*		
956		—	—	—	0 8
996		>4 0	8 0	<2 0	0 3
827		>2 0	<2 0	<1 0	1 0
839		0 8	<4 0	<5 0	0 5
849		—	—	—	0 3

TABLE VIII—*Concluded*

CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm / kgm	*mgm / kgm*		
828		—	—	—	0.05
841		>0.4	<0.8	<2 0	0.25

considerably less potency. Introduction of only one long alkyl radical was not as deleterious as introduction of two; for example, #764, with a methyl and an n-heptyl group on the tertiary nitrogen, retained an "*in vitro* index" of 0.5.

Number K-188 contained no tertiary nitrogen, and hence there was no resonating system between two nitrogens. This compound had an "*in vitro* index" of 0.02 which was low relative to the cyanines, but it was remarkable nonetheless, since it was the only compound studied which had an index of any significance despite the lack of the structural requirements described in the following paragraphs.

As has already been pointed out in previous communications (1, 2, 3), a high degree of antifilarial activity was observed with compounds possessing the resonating amidinium ion system, in which a quaternary nitrogen was separated from a tertiary nitrogen by a chain of atoms referred to as a "conjugated" chain, i.e., a chain whose members were joined by alternating single and double bonds. Tables I to X illustrate that marked antifilarial activity *in vitro* was not restricted to any particular ring or rings, and was observed regardless of whether both nitrogens (tables to IX) or only one nitrogen (table X) was a part of a heterocyclic ring. If neither of the two nitrogens was part of a heterocyclic ring (table IX: #K-218), activity could be present, although at a much lower order of magnitude. On the other hand, if all the atoms linking the two nitrogens were part of a chain, and not a portion of a heterocyclic ring, activity

in vitro was completely abolished (table IX: #K-217). In the case of one compound, only one of the three linking atoms was part of the heterocyclic ring, yet antifilarial activity was retained (table IV: #831). In almost every case the atoms interposed between the two nitrogens were carbon; that this was not a requirement for activity is indicated by the fact that nitrogen could serve in place of carbon, as in #849 of table VIII which had an "*in vitro* index" of 0.3.

Compounds closely related to cyanines, but lacking an amidinium ion resonating system exhibited either a very low degree of antifilarial activity *in vitro* or none at all (#797 and #821, table I; #854 and #855, table III; #945 and #946, table IV; #912, #925, #1001, and #921, table XI). The last mentioned group of compounds was interesting also from the standpoint of being possible degradation products of the cyanines *in vivo*. It has been pointed out already that in the case of one compound (K-188, table X), some activity was observed in spite of the lack of a resonating amidinium ion system. This compound contained a quaternary nitrogen, but lacked the tertiary nitrogen to complete the structural requirements outlined.

A large number of well-known organic dyes resemble the cyanines chemically in their possession of a tertiary and a quaternary nitrogen separated by a "conjugated" chain of carbon atoms. Since the antifilarial action of the cyanines appeared to depend on these particular structural features, it seemed of interest to include some non-cyanine dyes in the study for purposes of comparison. None of these was found to be active *in vivo*, but a considerable number possessed a low degree of activity *in vitro*. For the sake of brevity these non-cyanine dyes are listed here without their structural formulae; in each case the "*in vitro* index" is given within parentheses: (a) triphenylmethane dyes: crystalviolet (0.025), brilliant green (0.1), malachite green (0.1), fuchsin (rosaniline) (0.1); (b) phenazothionium dyes: methylene blue (0), azur B (0); (c) phenazinium dyes: janus green (0.1), diethylsafranin (0.05), safranin-T (0.025); (d) other dyes: acridine orange (0.01), meldolas blue (0.003), shodamine B (0.0025), methylquinoline yellow (>0.002). Fluorescein, which possesses a "conjugated" chain between two oxygen atoms instead of two nitrogen atoms, exhibited no antifilarial activity *in vitro*.

Because some organic antimony compounds have been shown to exhibit antifilarial activity *in vivo* (4, 5, 7, 11), 'Fuadin' and potassium antimonyl tartrate were tested *in vitro*. They were only $\frac{1}{10}$ to $\frac{1}{15}$ as active as the reference cyanine (#348) in regard to their ability to inhibit the oxygen uptake of *L. carinii*.

In contrast to their inhibitory effect on the respiration of malaria parasites (9), quinine and atabrine were found not to interfere with the respiratory metabolism of *L. carinii*. This is in agreement with the observation that these two antimalarials have no chemotherapeutic action *in vivo* against *L. carinii* in the cotton rat.

F. Selection of compounds for further study. It has already been pointed out that the inherent antifilarial activity of these compounds would operate in the infested animal host in proportion to the influence exerted by

TABLE IX

The relative antifilarial activity of a number of miscellaneous cyanine dyes against L. carinii of the cotton rat

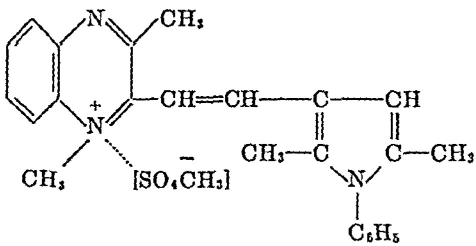
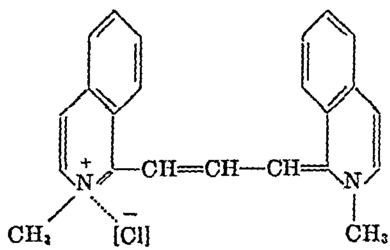
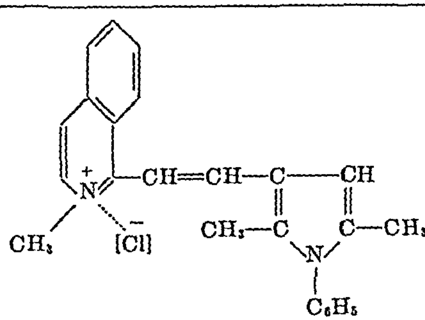
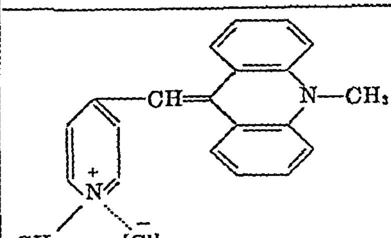
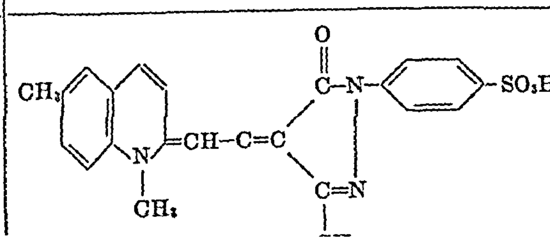
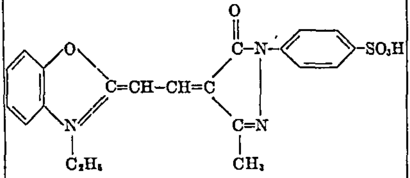
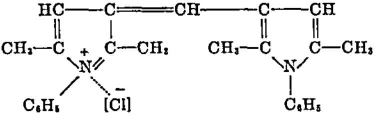
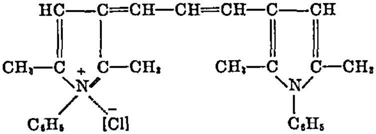
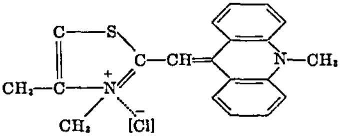
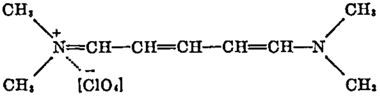
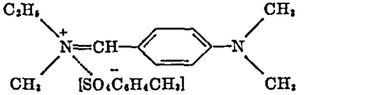
CHEMOTHERAPY CENTER NO.	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm./kgm.	*mgm./kgm.*		
830		>16.0	<16.0	<1.0	0.2
1021		>16.0	16.0	<1.0	0.3
1025		1.6	6.0	4.0	0.7
886		>8.0	8.0	<1.0	0.25
945		>20.0	>40.0	—	0.003

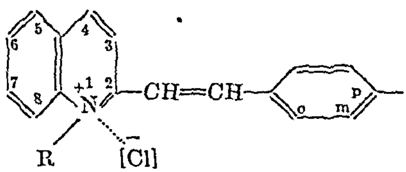
TABLE IX—*Concluded*

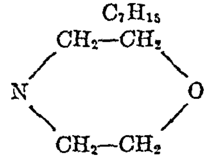
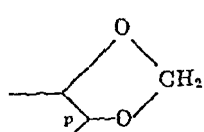
CHEMOTHERAPY CENTER NO	STRUCTURE	MINIMUM CURATIVE DOSE	MAXIMUM TOLERATED DOSE	THERAPEUTIC INDEX	IN VITRO INDEX
		mgm./kgm.	mgm./kgm.		
946		>16.0	>64.0	—	0
1113		>12.0	12.0	<1.0	0.2
1114		>16.0	16.0	<1.0	0.01
887		—	—	—	0.125
K217 (PD423)		—	—	—	0
K218		—	—	—	0

various host factors such as rate and extent of excretion, metabolic alteration, favorable and unfavorable distribution to the sites of occurrence of the invading parasite, etc. Since human filariasis involves a different host from that used

TABLE X

The relative antifilarial activity in vitro of a number of styryl-quinoline dyes against L. carinii of the cotton rat



CHEMOTHERAPY CENTER NO.	SUBSTITUENT GROUPS			IN VITRO INDEX
	R	p	6	
350	C_2H_5	$N(C_2H_5)_2$	CH_3	2.0*
759	CH_3	$N(C_3H_7)_2(n)$	CH_3	0.4
763	CH_3	$N(C_3H_7)_2^{(iso)}$	CH_3	1.0
760	CH_3	$N(C_4H_9)_2$	CH_3	0.3
762	CH_3	$N(C_5H_{11})_2$	CH_3	0.1
764	CH_3	CH_3 N C_7H_{15}	CH_3	0.5
757	CH_3		CH_3	0.5
773	CH_3	$N(C_2H_5)_2$	CH_3 C_2H_5—C— CH_3	1.0
K-188 (PD-245)	CH_3		—	0.02

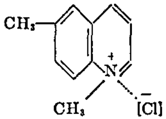
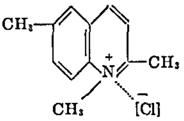
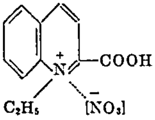
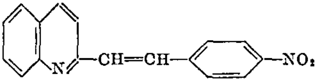
* *In Vivo:* Min. Cur. Dose = 1.6 mgm./kgm.
Max. Tol. Dose = <8.0 mgm./kgm.
Therapeutic Index = <5.0.

in this study, and a different site of occurrence of the invading parasite, namely the lymphatic system, it is obvious that the only infallible method of selecting the best compound would involve the testing against the human disease of at least those compounds shown to have a high degree of antifilarial action *in vitro*.

The impracticability of such a procedure is obvious. To minimize the modifying factors which might be imposed by a different host, and a different site of occurrence of the invading parasite, the compound selected from this study for further investigation should possess a high degree of activity against *L. carinii*, *both in vitro* and *in vivo*. Compound #863 (table IV) appeared to possess the best combination of indices, since it was twice as active as the reference compound (#348) *in vitro* and, in addition, had a high (absolute) therapeutic index. However, since many other qualifications would be demanded of a compound

TABLE XI

The relative antifilarial activity in vitro of a number of possible degradation products of cyanine dyes against L. carinii of the cotton rat

CHEMOTHERAPY CENTER NO.	STRUCTURE	IN VITRO INDEX
912		0
925		0
1001		0.001
921		0.003

to be administered to human beings, it seemed unwise to eliminate all but one compound at this early stage of the investigation. Furthermore, 12 compounds had therapeutic indices of 10 to 15. Only 4 of these showed less than 50 per cent of the activity of #348 *in vitro*, while 5 were more active than the reference compound. These compounds were numbers 712, 713, 798, 824, 835, 871, 863, 943, 963, 964, 965 and 967. All these were retained temporarily for further study in regard to possible therapeutic utility in human filariasis, with the exception of #712. This compound was omitted because of its very low solubility which accounts at least partly for its low degree of antifilarial activity

in vitro. This property also rendered an accurate measure of its chemotherapeutic activity impossible, since suspensions, rather than solutions, were injected, and residual drug was invariably found in the peritoneal cavity at autopsy following the administration of large doses.

An additional factor, unanswered by any of the above considerations, but of paramount importance in transferring a drug of this group from the laboratory to the clinic, was its relative antifilarial potency for *L. carinii* of the cotton rat and for *W. bancrofti* of man. This would depend largely on the degree of similarity or dissimilarity in the metabolic characteristics of these two parasites. The impossibility of obtaining *W. bancrofti* for studies *in vitro* made it necessary to postpone the appraisal of this critical factor until the time of clinical trial of the particular cyanine dye eventually selected for this purpose.

SUMMARY AND CONCLUSIONS

(1) In a routine "screening" program, the cyanine dye, (1-amyl-2,5-dimethyl-3-pyrrole)(1,6-dimethyl-2-quinoline) dimethincyanine chloride (Chemotherapy Center #348) was found to possess marked chemotherapeutic properties against the filarial parasite, *Litomosoides carinii*, of the cotton rat.

(2) In doses which are only a fraction of those maximally tolerated this compound was able to produce complete cures when administered intraperitoneally every eight hours for 6 days, once daily for 5 days, once daily for 3 days. Although a cure could be obtained with a single dose, the size of the latter approached that of the acutely lethal dose.

(3) This compound inhibited the oxidative metabolism of adult *L. carinii*, in dilutions as high as 1:40 million.

(4) A large number of related cyanine dyes were studied with regard to both their curative activity and their antimetabolic effect. The particular chemical structure which appears to be essential for the antifilarial effect, and the effect of various structural modifications on antifilarial activity, are discussed.

(5) On the basis of a high degree of antifilarial activity both *in vivo* and *in vitro*, eleven compounds were selected for further study with the object of an eventual selection of one for clinical trial in human filariasis.

REFERENCES

1. Welch, A. D., Peters, L., Bueding, E., Valk, A. D., Jr., and Higashi, A.: Science, 105: 486, 1947.
2. (a) Welch, A. D., Peters, L., Bueding, E., Valk, A. D., Jr., and Higashi, A.: Fed. Proc., 6: 382, 1947.
   (b) Peters, L., Higashi, A., and Welch, A. D.: Fed. Proc., 6: 362, 1947.
   (c) Valk, A. D., Jr., Peters, L., and Welch, A. D.: Fed. Proc., 6: 379, 1947.
   (d) Bueding, E.: Fed. Proc., 6: 312, 1947.
3. (a) Bueding, E.: Annals N. Y. Acad. Sciences, 50: 115, 1948.
   (b) Peters, L.: Annals N. Y. Acad. Sciences, 50: 117, 1948.
4. Culbertson, J. T., and Rose, H. M.: This Journal, 81: 189, 1944.
5. Culbertson, J. T., and Price, E.: This Journal, 87: 181, 1946.
6. Culbertson, J. T., Rose, H. M., and Oliver-Gonzalez, J.: Am. J. Trop. Med., 25: 271 and 403, 1945.

7 CULBERTSON, J T Annals N Y Acad Sciences, 50: 73, 1948
8 (a) BIETER, R N , CUCKLER, A C , LITCHFIELD, J T , JR , BREY, T E , AND WRIGHT, H N Fed Proc , 6 310, 1947
(b) WRIGHT, H N , CUCKLER, A C , CRANSTON, E M , AND BIETER, R N Fed Proc , 6: 388, 1947
(c) CRANSTON, E M , CUCKLER, A C , LITCHFIELD, J T , JR , BREY, T E , WRIGHT, H N , AND BIETER, R N Fed Proc , 6: 318, 1947
9 WRIGHT, H N , LITCHFIELD, J T , JR , BREY, T E , CRANSTON, E M , CUCKLER, E M , CUCKLER, A C , AND BIETER, R N Annals N Y Acad Sciences, 50. 115, 1948
10 OTTO, G F , AND MAREN, T H Annals N Y Acad Sciences, 50: 39, 1948
11 BROWN, W H Annals N Y Acad Sciences, 50 51, 1948
12 BROOKER, L G S Advances in Nuclear and Theoretical Organic Chemistry R E Burk and Oliver Grummitt Interscience Publishers, Inc , New York, N Y 1945, Chapter IV, pp 63–136
13 BROOKER, L G S Annals N Y Acad Sciences, 50 108, 1948
14 SILVERMAN, M , CEITHAML, J , TALIAFERRO, L G , AND EVANS, E A , JR Jour Inf Dis , 75. 212, 1944
15 BUEDING, E Jour Exp Med , 89: 107, 1949
16 PETERS, L , BUEDING, E , VALK, A D , JR , HIGASHI, A , AND WELCH, A D To be published

A PHARMACOLOGIC COMPARISON OF HEXAETHYL TETRAPHOSPHATE (HETP) AND TETRAETHYL PYROPHOSPHATE (TEPP) WITH PHYSOSTIGMINE, NEOSTIGMINE AND DFP[1,2]

PAUL R. SALERNO AND JULIUS M. COON

Toxicity Laboratory and the Department of Pharmacology, The University of Chicago, Chicago, Illinois

Received for publication October 29, 1948

The parasympathomimetic properties of a number of alkyl polyphosphate compounds have been established in the course of several investigations into the pharmacologic actions of these agents. DuBois and Mangun (1) were the first to report the cholinesterase inhibiting effect of hexaethyl tetraphosphate (HETP). They found that HETP was about four times as potent as diisopropyl fluorophosphate (DFP) in inhibiting rat brain cholinesterase activity *in vitro*. Hagan and Woodard (2) studied the toxicity of HETP for several species. Deichmann and Witherup (3) reported that tetraethyl pyrophosphate (TEPP) was about two and a half times as toxic as HETP when given orally to rats. Mangun and DuBois (4) included TEPP in a comparison of the potency of these compounds with that of DFP in inhibiting rat brain cholinesterase *in vitro*. They found that a 50 per cent inhibition was effected by 4×10^{-9} *M* TEPP, by 1.6×10^{-8} *M* HETP, and by 6.3×10^{-8} *M* DFP under the same conditions. Roeder and Kennedy (5) compared the effects of stimulating the afferent nerves entering the sixth abdominal ganglion of the cockroach in the presence of 6×10^{-5} *M* DFP and of 2.5×10^{-7} *M* HETP. They observed similar changes consisting of prolonged after discharge in the giant fibers of the ventral nerve cord and ganglionic transmission alternating from facilitation to temporary block. Koppanyi, Karczmar and King (6) gave evidence that TEPP increases the sensitivity of sympathetic ganglia at certain dosage levels. DFP and physostigmine were shown to have similar actions. Burgen *et al.* (7) compared the effects of TEPP and HETP on isolated tissues and on the chloralosed cat. Dayrit, Manry, and Seevers (8) made some detailed observations on the pharmacologic action of HETP with special reference to its cardiovascular, respiratory, and anticholinesterase effects in dogs.

The purpose of this study was to compare directly, under the same experimental conditions, some of the pharmacodynamic actions of HETP and TEPP with those of three other better known cholinesterase inhibitors, e.g., physostigmine, neostigmine, and DFP, in order to add to the information which might form the basis for selecting from these agents one which would serve best in

[1] Preliminary report, Fed. Proc., 7: 212, 1948.

[2] This investigation was supported in part by the Office of Naval Research, under contract N6 ori-20, Task Order 11; and in part under contract between the Medical Division, Chemical Corps, U. S. Army and the University of Chicago Toxicity Laboratory. Under the terms of this contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

specific cases as a research tool or as a therapeutic agent. The comparative actions of these five anticholinesterase agents were observed on the blood pressure, electrocardiogram, isolated heart, and small intestine of various species of experimental animals.

EXPERIMENTAL. *Effects on Blood Pressure.* Forty-six cats weighing 2 to 4 kgm. and 25 dogs weighing 7 to 12 kgm. were used in this study. The cats were anesthetized with 180 mgm./kgm. and the dogs with 150 mg./kgm. of Na-phenobarbital intraperitoneally. The trachea was cannulated in all animals and a Palmer positive pressure artificial respiration pump was used when required. Blood pressure changes were recorded by a mercury manometer connected to the right carotid artery. The right femoral vein was used for injection. Chest movements were recorded by means of a tambour actuated by a pneumograph placed on the chest.

A single intravenous injection of 0.3 mgm./kgm. HETP, 0.1 per cent in saline, generally produced a 30 to 40 mm. rise in blood pressure which reached its maximum in 1 to 2 minutes and subsided to normal in 7 to 10 minutes. This effect was not accompanied by any significant change in heart rate, or in respiratory rate or amplitude. A second injection of the same dose, given 15 minutes to 4 hours after the first, produced a depressor response of about the same magnitude and duration (fig. 1). A third such dose also elicited a fall in blood pressure, this time attended by a marked slowing of the heart and a fatal respiratory paralysis (fig. 1). Atropinized cats (1 mgm./kgm.) gave successive pressor responses to these doses of HETP and survived three to five times the amount tolerated by unatropinized animals. Larger doses of atropine (10 mgm./kgm.) afforded greater protection against HETP toxicity and enhanced the rise in blood pressure. The responses to 0.1 mgm./kgm. of TEPP or neostigmine and 0.2 mgm./kgm. of physostigmine were equivalent to those to 0.3 mgm./kgm. of HETP under these conditions. Furthermore, these drugs could be used interchangeably in a series of injections eliciting the above described sequence of blood pressure changes, i.e. using the above doses the pressor response to any one was followed by a depressor response to the subsequent injection of another. The blood pressure changes produced by DFP alone were slight and variable, but 0.6 mgm./kgm. of DFP would reverse the pressor response to the subsequent injection of any of the above agents.

When smaller doses of HETP (0.15 mgm./kgm.) were injected intravenously, successive rises of 10 to 15 mm. blood pressure could be elicited until the fourth or fifth injection which resulted in a depressor response. This sequence also resulted from comparable doses of TEPP, neostigmine, or physostigmine and here again these drugs could be used interchangeably in the series of injections.

The injection of 0.6 mgm./kgm. or more of HETP produced first a 30 to 40 mm. rise in blood pressure lasting 2 to 3 minutes followed by a precipitous fall accompanied by a marked slowing of the heart and a great increase in pulse pressure suggesting strong vagal action with A-V block (fig. 2). This picture persisted for as long as 30 minutes and artificial respiration was frequently required to prevent death. This change was not influenced by sectioning both vagi but

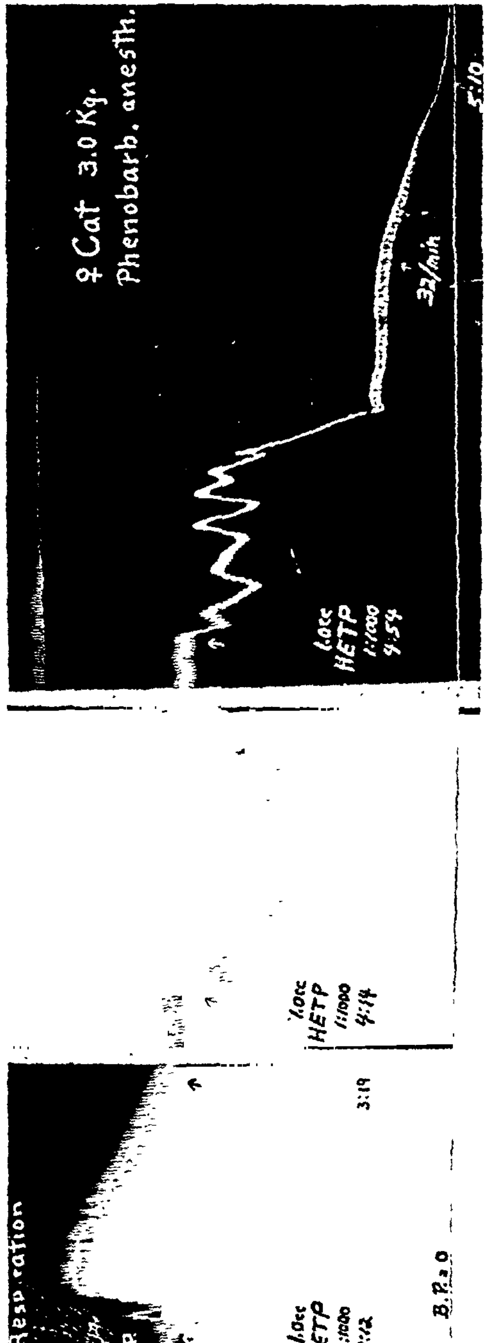


FIG. 1. BLOOD PRESSURE RESPONSES TO SUCCESSIVE DOSES OF 0.33 MGM./KGM. OF HETP SHOWING PRESSOR RESPONSE FROM FIRST INJECTION AND DEPRESSOR RESPONSES FROM SECOND AND THIRD INJECTIONS

Note time intervals and terminal respiratory paralysis

was abolished by 1.0 mgm./kgm. of atropine. The approximate threshold doses required to elicit this response were 0.2 mgm./kgm. of TEPP or neostigmine, 0.4 mgm./kgm. of physostigmine and 1.0 mgm./kgm. of DFP. In the case of DFP this action was without the preliminary rise in blood pressure and did not occur until about ten minutes after injection. Two successive doses of 0.3 mgm./kgm. of HETP potentiated the depressor action of acetylcholine about ten-fold. Similar potentiation was produced by comparable doses of each of the other four drugs tested.

The pressor effects of physostigmine and neostigmine have never been satisfactorily explained (9, 10, 11). Experiments were therefore conducted to attempt

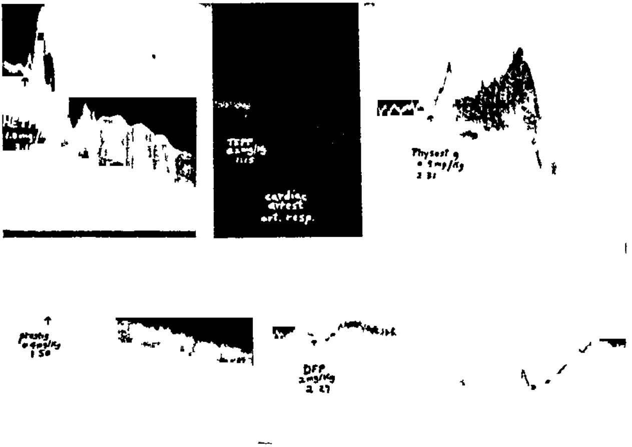


FIG. 2. BLOOD PRESSURE RESPONSES IN ANESTHETIZED DOGS (SODIUM PHENOBARBITAL) TO SINGLE LARGE DOSES OF EACH OF FIVE CHOLINESTERASE INHIBITORS STUDIED

Showing marked primary pressor responses (except with DFP), and precipitous fall in blood pressure and great increase in pulse pressure (strong vagal action). Note delayed action of DFP and restorative effect of 20 mgm./kgm. of procaine.

to elucidate the mechanisms involved in these blood pressure changes. Complete nicotinization of the animal by giving a series of injections of nicotine sulfate at short intervals (1 minute or less) until the pressor response to this drug was completely abolished did not influence the pressor responses to HETP, TEPP, neostigmine or physostigmine. This indicated that any ganglionic effect was not nicotine-like. These drugs also elicited their typical pressor responses in adrenalectomized animals. Bilateral nephrectomy, vagotomy, evisceration or decapitation also had no definite effect on the pressor responses.

Small doses of the sympatholytic agent dibenamine HCl (5.0 mgm./kgm.), which reverse the pressor effect of injected epinephrine, did not influence the

pressor response to HETP or nicotine. However, large doses of dibenamine HCl (30 mgm./kgm.) reversed the pressor response to both these drugs. This difference in the effects of adrenolytic and sympatholytic doses of dibenamine further supports the conclusion that liberated epinephrine does not contribute markedly to the pressor responses to these drugs.

It is known (12) that the injection of procaine produces significant changes in the excitability of the autonomic nervous system. The depressor response to injected acetylcholine is greatly reduced, the pressor response to nicotine is reduced and the pressor response to epinephrine is increased. In a dose of 20 mgm./kgm. procaine was seen to exert an atropine-like effect in preventing the depressor effects of HETP and TEPP and in abolishing the bradycardia and restoring a normal heart action after the larger doses of these drugs. Procaine was much less effective than atropine in this respect and the duration of its restorative and the extent of its protective actions were much less than that of atropine.

Haimovici and Pick (13) reported that thiamine blocks the pressor action of nicotine. We found that 200 mgm./kgm. of thiamine-HCl given slowly in divided doses would effectively block nicotine and would markedly suppress the pressor responses to HETP and TEPP. Thiamine also blocks the stimulating effect of nicotine on striated muscle as shown on the frog rectus abdominis by Unna and Pick (14). They concluded that this action of thiamine was on the myoneural junction.

Electrocardiographic Changes. The Sanborn string galvanometer electrocardiograph was employed on 22 dogs. In order to obtain suitable records consistently the dogs were anesthetized with 150 mgm./kgm. of Na-phenobarbital. Observations were made using the conventional Leads I, II, and III.

Electrocardiograms taken following single intravenous doses (fig. 3) of the drugs being compared revealed that similar profound changes in cardiac rhythm were produced by all of the agents. Electrocardiographic findings following HETP have been described by Dayrit, Manry, and Seevers (8) who observed a marked sinus bradycardia without an increase in the P-R interval, a partial to complete A-V block, and with larger doses a disappearance of the P waves. In addition to these changes we regularly observed, with the five drugs studied, marked changes in the T wave, such as inversion in all leads, or exaggeration with or without inversion especially in leads II and III. On one occasion inversion was seen in alternating cardiac cycles in all three leads after TEPP. Small doses were capable of producing a pressor effect with no visible electrocardiographic changes. Moderate doses produced a slowing which sometimes developed into varying degrees of A-V block. Large doses frequently precipitated a sudden A-V block without preliminary slowing, and in this case the P wave often disappeared and there was an increase in the magnitude and variability of the T wave.

The A-V block was manifested in almost every possible way. A-V ratios of 2:1 and 3:1 were frequently seen. More commonly however such blocks soon developed into complete A-V dissociation with approximate ratios ranging from

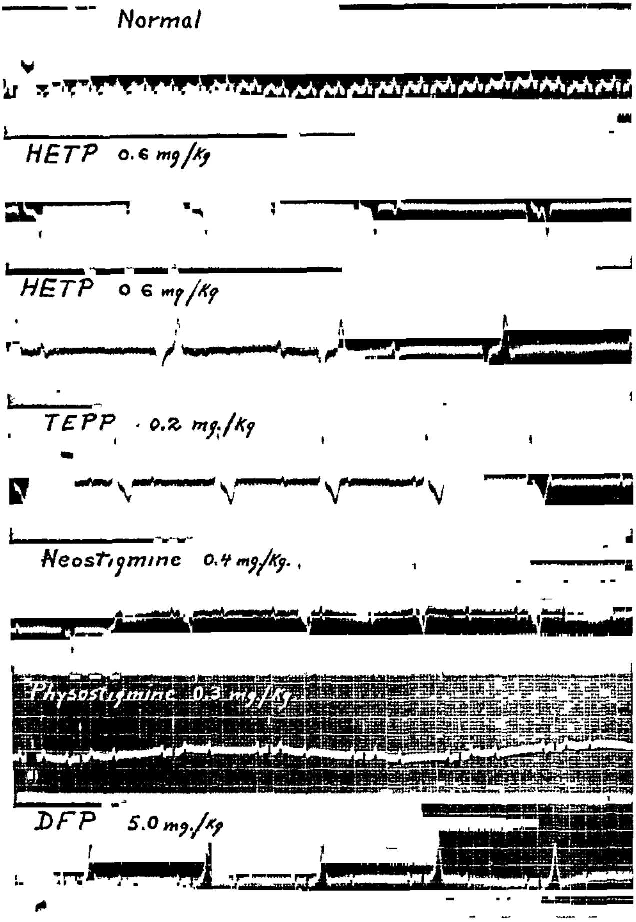


FIG 3 ELECTROCARDIOGRAMS OF DOGS ANESTHETIZED WITH SODIUM PHENOBARBITAL
Leads indicated by white blocks at upper edge of each record

2 1 to 5:1 There was no evidence that there was any difference in the cardiac effects of HETP, TEPP, physostigmine, neostigmine, and DFP, except for the

delayed onset of the action of the DFP. Since these changes were not affected by vagotomy but were prevented or abolished by atropine or procaine and prevented by thiamine given previously, it is suggested that the site of action is at or peripheral to the intrinsic synapses of the parasympathetic innervation of the heart. Since the isolated heart was found to be highly resistant to all these anticholinesterase agents (see below) it appears likely that the observed effects on the heart of the intact animal are due to an accumulation of acetylcholine coming to the heart by way of the circulation.

Effects on the Isolated Rabbit Heart. Experiments were conducted upon 40 isolated rabbit hearts perfused with oxygenated Ringer-Locke solution at a pressure of 50 to 70 cm. of perfusion solution at 38°C. The aorta was cannulated and the perfusion fluid forced by gravity into the coronary arteries. Observations were made on the effects of the anticholinesterase agents administered (a) as single injections into the perfusion line immediately adjacent to the heart and (b) in various concentrations in the stock perfusion fluid.

The results of these tests indicated that the isolated heart exhibits little specific sensitivity to HETP or TEPP, as has been reported for physostigmine (15), DFP (16), and neostigmine (9). The injection of 1.0 mgm. (1.0 cc. of 1:1,000) of HETP directly into the cannula leading to the aorta and coronary circulation produced no apparent change in heart activity. In the intact cat or dog profound cardiac changes were always observed upon intravenous injection of 0.6 mgm./kgm. Following the injection of 5 mgm. (0.25 cc. of 1:50) of HETP, the amplitude and coronary flow of the isolated heart were reduced to about one-half normal for about three minutes without any marked change in rate. A good heart action was restored after this time but the amplitude did not return to normal (fig. 4). A second injection of the same dose produced the same reduction in amplitude but this time an increase in rate of coronary flow occurred. However, the heart did not recover from the second dose and heart action stopped in about 15 minutes. These changes in amplitude and coronary flow were not influenced in any way by the presence of atropine in the Ringer-Locke solution in a concentration of 2 mgm./liter (fig. 4). The responses of the isolated rabbit heart to TEPP, physostigmine, neostigmine, and DFP administered in this way were qualitatively the same as those described for HETP.

The maximum concentrations of HETP, TEPP, physostigmine, and DFP tolerated for one hour without effect on the isolated heart were determined when these agents were present in the oxygenated Ringer-Locke perfusion solution. These concentrations are shown in table 1 along with an estimate of the extent to which they reduce the amount of acetylcholine necessary to produce a minimal and evanescent slowing of the heart. These results show no apparent correlation between the maximum tolerated perfused concentrations and the reported cholinesterase inhibiting capacity or the toxicity of the various agents. Furthermore there appeared to be no relation between the tolerated concentrations and the degree of the potentiation of the response to acetylcholine.

The duration of the cardiac depression caused by minimal effective doses (0.2 cc. 1:50,000) by acetylcholine was increased about five times by previous treat-

ment with the anticholinesterase agents. The potentiation of the action of acetylcholine was less following temporary perfusion or single injections of sub-toxic doses of HETP than during continuous perfusion with the drug. However, the isolated heart from a rabbit sacrificed one day after receiving a

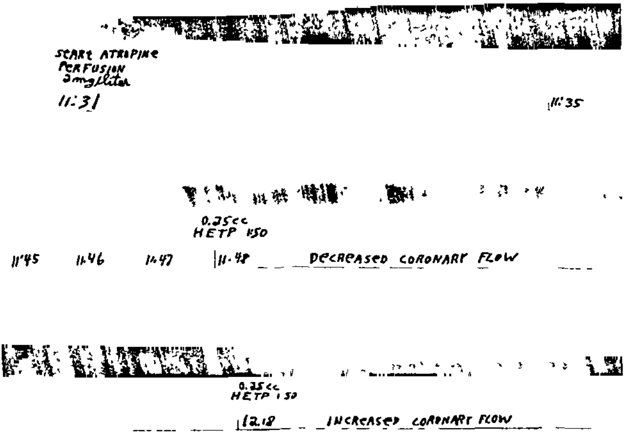


FIG. 4. ISOLATED RABBIT HEART PERFUSED WITH LOCKE-RINGER SOLUTION
Atropine did not influence the changes produced by HETP

TABLE 1

Maximum perfusion concentrations of anticholinesterase agents tolerated for one hour by isolated rabbit heart and resultant potentiation of the sensitivity to acetylcholine

ANTI CHOLINESTERASE AGENT	MAX TOLERATED CONCN $\times 10^{-4}$ M	APPROXIMATE ACETYL-CHOLINE POTENTIATION
DFP	0.38	40 times
HETP	2.0	40 times
TEPP	3.5	40 times
Physostigmine .	5.0	20 times

dose of 0.3 mgm./kgm. HETP showed about a 20-fold decrease in the dose of acetylcholine usually required to elicit a minimal response.

These studies indicated no qualitative differences in the actions of the five drugs used on the isolated rabbit heart. Relatively large amounts of each were required to produce what appeared to be a direct toxic effect. This action is considered totally independent of the anticholinesterase properties of the agents.

Effects on the Small Intestine of the Rabbit. The comparative actions of the

five agents under investigation were studied using two hundred and thirty washed strips of rabbit ileum suspended in oxygenated Ringer-Locke solution in 100 cc. glass baths kept at a constant temperature of 37.5°C. Parallel tests were usually carried out on two intestinal strips recording simultaneously. All tests were made using gut specimens removed from a freshly killed animal. In a few experiments the motility of the gut *in situ* was recorded by means of a device described by Jackson (17).

HETP, TEPP, neostigmine, physostigmine, and DFP produced qualitatively the same changes in the activity of the isolated rabbit ileum. The minimal concentration producing a characteristic gradual increase in tone and a delayed change from the normal pendular rhythm to a peristaltic type of rhythm was determined for each drug. A departure from the pendular activity occurred abruptly 15 to 20 minutes after the introduction of the drug into the bath and the new peristaltic rhythm was characterized by a series of profound relaxations and strong contractions alternating at regular intervals of 1 to 3 minutes (fig. 5). This peristaltic type of rhythm persisted as long as recordings were made, 3 to 4 hours in some cases. The reversibility of these changes of gut activity was tested by repeated washing. It was found that although these effects of physostigmine and neostigmine were easily reversed by washing, with a return to normal pendular activity, the effects of HETP, TEPP, and DFP could not be reversed by washing. These findings are summarized in table 2. The potency of these drugs on the rabbit gut was of the same order as their reported effectiveness as cholinesterase inhibitors (6). The effects of HETP on four intestinal strips from two guinea pigs were essentially the same as those observed on the rabbit ileum.

Atropine sulfate in a concentration of 3×10^{-8} M restored normal tone and activity following the onset of the changes induced by a minimal effective dose of each anticholinesterase drug (fig. 6A). The magnitude of the antagonizing molar concentration of atropine (10^{-8}) is of the same order as that of the minimal effective concentrations of all five agents. Larger doses of the cholinesterase inhibitors (100 to 1000 times the minimum effective dose) broke through the protection afforded by the above concentration of atropine but did not completely abolish the normal pendular rhythm. The fact that a given concentration of atropine blocked the action of a wide range of doses of a cholinesterase inhibitor probably indicates that the atropine is antagonizing endogenous acetylcholine which may be independent of the dose of the inhibitor over this range.

An increase above the minimal effective concentration of the anticholinesterase drug in the bath resulted in a more rapid onset of the typical changes in the rhythm of the intestinal strip. A one hundred-fold increase in concentration gave rise to an immediate effect. One-tenth the minimal effective dose of HETP potentiated the response of the gut to acetylcholine about 100 times. The administration of acetylcholine into the bath after a minimal effective dose of HETP hastened the onset of effect of the latter. Acetylcholine itself, however, did not induce the changes in rhythm described above for the cholinesterase inhibitors.

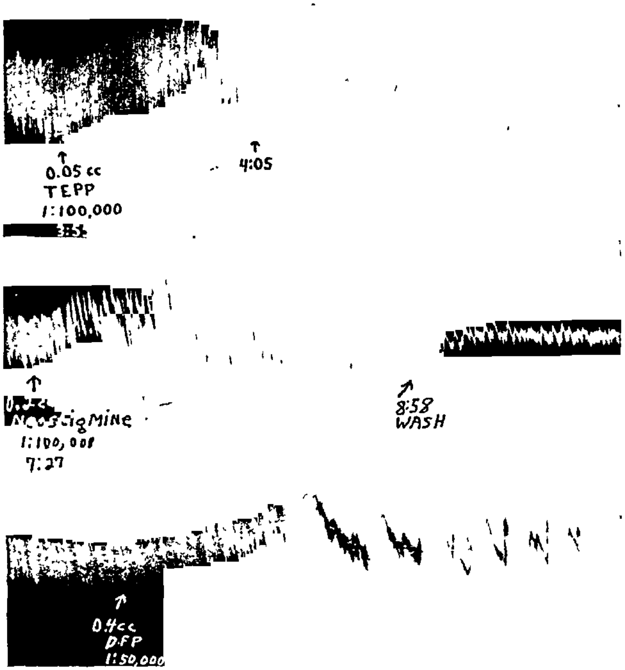


Fig. 5 Isolated Rabbit Ileum Suspended in 100 cc. Bath of Oxygenated Locke Ringer Solution to which the Above Indicated Doses were Added

Note slow rise in tone, delayed sudden change from pendular to peristaltic rhythm, and reversibility of the effect of neostigmine by washing.

TABLE 2

Minimal effective concentrations of anticholinesterase drugs producing changes in rhythm of isolated rabbit ileum, and the influence of washing

AGENT	MINIMAL EFFECTIVE CONCENTRATION $\times 10^{-8}$		IS EFFECT REVERSED BY WASHING?
	By Weight	On Molar Basis	
TEPP	1.0	3 5	No
Physostigmine .	1.4	3.5	Yes
Neostigmine.	1.4	4.3	Yes
HETP .	5 0	9.8	. No
DFP .	10 0	54.3	No

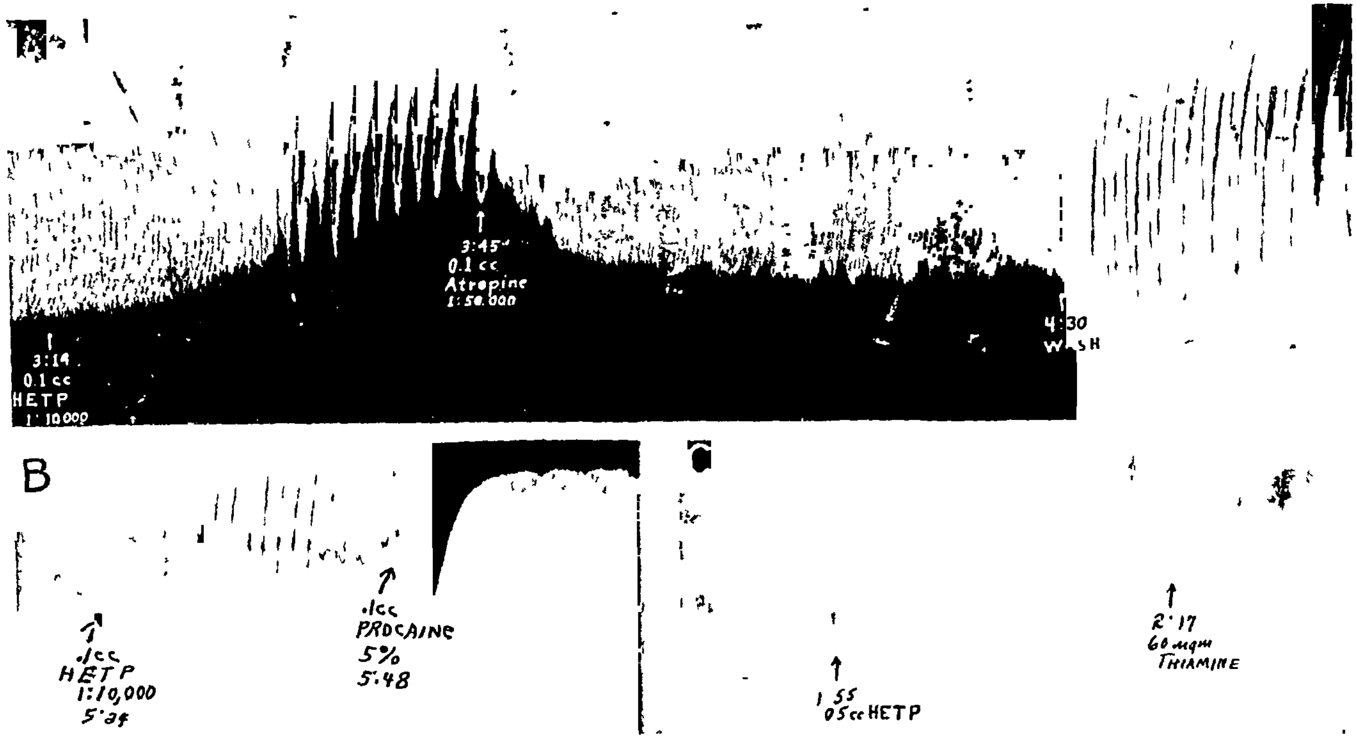


FIG 6 ISOLATED RABBIT ILEUM AS IN FIG 5

(a) The reversal to pendular rhythm caused by atropine was removed by washing. (b, c) Procaine and thiamine caused reversal to pendular rhythm but did not block rise in tone.

The nature of the response of the intestinal strip to these drugs was further studied in relation to nicotine, procaine, and thiamine A series of injections of nicotine (0 2 cc of 1 10,000 into a 100 cc bath) at about five minute intervals resulted in successively smaller fleeting increases in tone until at the end of the fourth injection the intestinal strip was no longer responsive Here it is supposed that the nicotine paralyzed nerve tissue only (ganglion cells), for the normal pendular activity, which is thought to be independent of nerve tissue, was not affected When an effective dose of HETP was then given, there resulted an increase in tone and a decrease in amplitude but not distortion of rhythm If a series of nicotine injections was introduced into the bath after the full HETP effect was produced, the pendular type of activity was restored

Procaine-HCl in a concentration of 5×10^{-6} maintained normal pendular activity if given before or restored it if given after an effective dose of any of the cholinesterase inhibitors studied (fig 6B) The action of procaine was like that of atropine except that the rise in tone was not blocked Procaine merely preserved the pendular rhythm Procaine in doses which had little effect on gut activity blocked the effects of nicotine and reduced the response to acetylcholine The significance of this will be discussed later

Thiamine-HCl in a concentration of 5×10^{-4} was also effective in reversing the effects of minimal effective concentrations of the cholinesterase inhibitors (fig 6C) Unna and Pick (18) have shown that thiamine will block the action of nicotine on the gut Following HETP, TEPP, and DFP the blocking actions of thiamine, atropine or procaine could be reversed by flushing out the bath, this reestablished the typical peristaltic rhythm previously induced by these agents This effect in the case of atropine is demonstrated in fig 6A

Small doses of HETP (less than 0 15 mgm /kgm) which did not elicit marked blood pressure responses produced a marked contraction of the small intestine *in situ* in the cat The increase in tone and increased peristaltic action under these conditions lasted only about 20 minutes Similar responses were observed with comparable doses of each of the other four cholinesterase inhibitors Apparently the distribution of the drug in the intact animal is such that the concentration attained in the intestine has only a fleeting action

Preliminary tests showed that the isolated uterus of the rabbit or guinea pig was not responsive to the drugs studied here

DISCUSSION The results of this comparison of five potent cholinesterase inhibitors discloses an extensive parallelism between these drugs with respect to certain pharmacologic actions With only one exception, namely the failure of DFP to elicit a consistent pressor response upon intravenous injection in the dog and cat, all five agents exhibited the same pharmacologic properties when tested upon the circulation of anesthetized dogs and cats, upon the electro cardiogram in the anesthetized dog, and upon the isolated heart and intestinal strip of the rabbit Since these five drugs gave qualitatively similar responses, it is not unlikely that a common site and mode of action is the causal factor in producing these responses in the physiologic systems tested The manner in which the effects are influenced by other pharmacologic agents and the fact that

these drugs are interchangeable in eliciting the characteristic sequence of blood pressure responses with repeated doses in the intact animal constitutes further support for a common mode of action.

It is readily conceivable that in the case of the isolated intestine, this mode of action is based solely on the anticholinesterase activity of the drugs. Here it seems significant that the minimal effective concentration of these agents was of the same order as the reported 50 per cent cholinesterase inhibitory concentrations (6). In the case of the isolated heart, however, where there is no accumulation of acetylcholine, the observed effects of these cholinesterase inhibitors could hardly be attributed to an inactivation of cholinesterase. Their actions here were still markedly similar, however, though the amounts necessary were so large that the effects could not possibly be of significance in intact animal studies.

The pressor effects of these agents have been described (6, 9, 10, 11) but they have not been satisfactorily explained. Koppanyi *et al.* (6) offer evidence that the cholinesterase inhibitors alter the sensitivity of sympathetic ganglia. In confirmation of the results of other workers we were not able to diminish significantly the responses to these drugs by adrenalectomy or evisceration. Nicotinization also had no influence. However, we succeeded in reversing the pressor effects of HETP, TEPP, physostigmine and neostigmine by large sympatholytic doses of dibenamine. Smaller adrenolytic doses of dibenamine did not modify the pressor responses. These facts constitute evidence that the drugs either are able to stimulate sympathetic ganglia which are presumably paralyzed by nicotine, or can exert a peripheral sympathin-like action which requires large doses of dibenamine to neutralize. Neither of these possible actions could be considered referable to an inhibition of cholinesterase.

A very rapidly developed apparent tachyphylaxis to the pressor action of these agents was observed. This was not a true tachyphylaxis, however, for the atropinized animal gave successive pressor responses to repeated moderate doses until a respiratory paralyzing dose accumulated in the body. It is logical to suppose that small pressor doses do not have sufficient anticholinesterase activity to result in any obvious parasympathetic stimulation, but with repeated doses the cumulative antiesterase action becomes dominant and any persisting peripheral vasoconstriction is masked.

Why DFP does not elicit a pressor response like the other drugs studied is not known. Some light on this question may be furnished by the fact that DFP has a greater affinity for the non-specific plasma cholinesterase than for the specific true cholinesterase of brain and peripheral nerve (21, 22). It is well known that very low plasma esterase levels are not necessarily reflected in pharmacodynamic responses. It is significant that DFP, which does not exhibit a pressor action, readily contributes to the induction of tachyphylaxis to the pressor action of HETP, TEPP, physostigmine and neostigmine.

In the intact animal the cardiac effects of the agents studied may be explained by the accumulation of acetylcholine. Since these effects are not influenced by vagotomy but are abolished or prevented by atropine, and do not occur in the

isolated heart, one must conclude that the accumulated acetylcholine in this case comes to the heart by way of the circulation, and is not the product of a stimulation of the parasympathetic ganglia in the heart muscle by the antiesterases themselves. It is possible, however, that the acetylcholine so accumulated may induce such a ganglionic stimulation, thus resulting in the liberation of more acetylcholine at the nerve endings. The comparatively weak blocking action of procaine, compared with that of atropine, against the cardiac effects of the esterase inhibitors, may be explained on this basis. It may be presumed that procaine eliminates the action of acetylcholine on the synapses but affects to a lesser extent (19) the action on the cardiac effector cell.

In the case of the isolated rabbit heart it was observed that the maximum concentrations of the antiesterase drugs tolerated for one hour without effect were of the order of 10,000 times more than the minimal concentrations which would produce an increase in tone and a change in rhythm of the isolated rabbit intestine. This difference would be expected from the fact that acetylcholine is continually being formed in the isolated gut (23) but not in the isolated heart. The atropine-like action of procaine and thiamine in reestablishing a normal pendular rhythm after its distortion by the esterase inhibitors suggests that ganglionic stimulation plays a role in the effects of the latter on the gut, since procaine and thiamine are thought to interfere with nerve transmission but not with the action of acetylcholine on the effector cell. It is supposed that such a ganglionic stimulation results from the accumulated acetylcholine and not directly from the esterase inhibitor itself. It is significant that nicotine, in concentrations sufficient to paralyze the ganglion cells, prevents the typical change in rhythm induced by the antiesterases. We were not able to elicit this change with acetylcholine itself, possibly due to the failure of this agent to gain access to the exact site at which the physiologically liberated acetylcholine acts.

The mechanism by which the anticholinesterase agents abolished the pendular movements in the rabbit gut and set up a slow peristaltic rhythm is not clear. The fact that the pendular activity can continue in the presence of atropine, procaine, thiamine, and nicotine suggests that this behaviour is independent of neurogenic factors. These drugs, however, readily prevent or abolish the peristaltic activity induced by the antiesterases, suggesting that this behaviour is on a neurogenic basis and depends on the accumulation of acetylcholine. It is conceivable that the onset of the peristaltic action, accompanied by strong constriction rings in the circular muscle, interferes with the contractions of the longitudinal muscle which constitute the pendular movements.

It was observed (table 2) that TEPP, physostigmine, and neostigmine were approximately equal in potency with respect to the minimal molar concentrations producing a change in rhythm in the isolated rabbit gut, while HETP and DFP were less effective. It is of interest to note that Koppanyi *et al.* (6), using a pharmacodynamic response in the dog to estimate the concentrations of TEPP, physostigmine, HETP, and DFP necessary to produce 50 per cent inhibition of cholinesterase arrived at the same relative potencies for these agents as were

derived from our experiments. Though our absolute concentrations were of the order of 100 times higher than those tabulated by Koppanyi *et al.*, they are approximately equivalent to the 50 per cent esterase inhibiting concentrations measured directly on rat brain and cockroach tissue (1) and considerably lower than similar measurements on human and horse serum (20). To what extent the isolated rabbit intestine might be useful in the bioassay of the cholinesterase inhibiting activity of chemical substances must be a subject of further investigation.

SUMMARY

1. The pharmacological effects of HETP and TEPP were compared with those of physostigmine, neostigmine and DFP on the blood pressure of anesthetized cats and dogs, on the electrocardiogram of anesthetized dogs, and on the isolated heart and intestine of the rabbit.

2. In these tests HETP and TEPP exhibited no qualitative differences in action from physostigmine and neostigmine.

3. With the exception of DFP, all of the drugs compared elicited qualitatively similar cardiovascular responses. DFP lacked a definite pressor effect and the onset of electrocardiogram changes was delayed.

4. Typical electrocardiographic changes following each drug were bradycardia, A-V block and dissociation, exaggeration and inversion of the T wave, and disappearance of the P wave.

5. The isolated rabbit heart was relatively insensitive to high perfusion concentrations or to single injections. Effective concentrations of each drug caused depression in amplitude without change in rate. The order of potency in this respect was DFP > HETP > TEPP > physostigmine.

6. The isolated rabbit intestine exhibited a high degree of sensitivity to all these agents. After a latent period the pendular movements were interrupted by a slow peristaltic type of activity. In their minimal effective concentrations these drugs bore the same relation to each other as in their reported anticholinesterase activities. Their effective concentrations were also of the same order as the 50 per cent cholinesterase inhibiting concentrations which have been reported.

7. These changes produced by HETP, TEPP, and DFP in the isolated intestine were not reversed by flushing the bath while the changes induced by physostigmine and neostigmine were reversed by flushing.

8. The tested pharmacologic actions of each of the five antiesterases were influenced in a similar way by other drugs, such as atropine, dibenamine, nicotine, procaine, and thiamine.

REFERENCES

1. DuBois, K. P., and Mangun, G. H.: Proc. Soc. Exper. Biol. & Med., 64: 137, 1947.
2. Hagan, E. C., and Woodard, G.: Fed. Proc., 6: 335, 1947.
3. Deichmann, W. B., and Witherup, S.: Fed. Proc., 6: 322, 1947.
4. Mangun, G. H., and DuBois, K. P.: Fed. Proc., 6: 353, 1947.
5. Roeder, K. D., and Kennedy, N. K.: Fed. Proc., 6: 191, 1947.

6. Koppanyi, T., Karczmar, A. G., and King, T. O.: Science, 106: 492, 1947.
7. Burgen, A. S. V., Keele, C. A., Chennells, M., del Castillo, J., Floyd, W. F., Slome, D., and Wright, S.: Nature, 160: 760, 1947.
8. Dayrit, C., Manry, C. G., and Seevers, M. H.: This Journal, 92: 173, 1948.
9. Mendez, R., and Ravin, A.: This Journal, 72: 80, 1941.
10. Stewart, G. N., and Rogoff, J. M.: This Journal, 17: 227, 1921.
11. St. Heathcote, R.: This Journal, 44: 95, 1932.
12. Hazard, R.: Presse Med., 53: 149, 1945.
13. Haimovici, H., and Pick, E. P.: Proc. Soc. Exper. Biol. & Med., 62: 234, 1946.
14. Unna, K., and Pick, E. P.: This Journal, 81: 294, 1944.
15. Greene, C. W., and Maneval, K. E.: This Journal, 46: 303, 1932.
16. Cohen, S. L.: Fed. Proc., 6: 89, 1947.
17. Jackson, E. E.: Experimental Pharmacology and Materia Medica, 2nd Edition, C. V. Mosby Co., 1939, p. 523.
18. Unna, K., and Pick, E. P.: This Journal, 81: 294, 1944.
19. De Elio, F. J.: Brit. J. Pharmacol., 3: 108, 1948.
20. Mazur, A., and Bodansky, D.: J. Biol. Chem., 163: 261, 1946.
21. Adrian, E. D., Feldberg, W., and Kilby, B. A.: Brit. J. Pharmacol., 2: 56, 1947.
22. Hawkins, R. D., and Mendel, B.: Brit. J. Pharmacol., 2: 173, 1947.
23. Dikshit, B. B.: Quart. J. Exp. Physiol., 28: 243, 1938.

TOXIC AND PATHOLOGIC EFFECTS OF XYLIDINE IN THE FASTING AND NON-FASTING STATES

SAMUEL S. SPICER, BENJAMIN HIGHMAN AND A. RALPH MONACO

Experimental Biology & Medicine Institute, Laboratory of Physical Biology, and Pathology Laboratory, National Institutes of Health, Bethesda, Maryland

Received for publication November 3, 1948

Although much is known of the toxicity of aromatic amino compounds, little is understood about factors which influence their effect *in vivo*. Claims have been made concerning the aggravating effect of alcohol on the methemoglobinemia of aromatic amines (1), and the protective effect of partial hepatectomy in aniline poisoning (2). In this report the advantage of the fasting over the postprandial state in withstanding exposure to vapors of commercial xylidine is demonstrated. Fasted cats were found to tolerate exposure to the xylidine-laden atmosphere for hours without evidence of serious disturbance. On the other hand, cats given large amounts of protein by forced feeding prior to exposure to xylidine exhibited a striking syndrome characterized by hyperpnea, panting, ptyalorrhea, agitation, and not infrequently death. Pathologic studies were made in search of a basis for this marked difference in reaction.

Procedure. The procedure adopted for exposing animals to xylidine by inhalation resembled that previously reported from this laboratory (3). Aliquots of a single shipment of xylidine recently received or purified by distillation were placed in the bubbler, and air was passed through this and on into a 400-liter capacity exposure chamber at a rate of about 20.6 liters per minute. This vapor was diluted with air from a line having a flow of 170 liters per minute, thus insuring adequate ventilation. By employing a commercial preparation of the 6 xylidine isomers, conditions which might actually occur were more nearly reproduced. Xylidine concentration in the chamber atmosphere was determined by collecting an air sample in dilute H_2SO_4, diazotizing and coupling with "H acid" (3).

Two to 6 cats, including at least one fasted and one yeast-fed animal (force-fed), were put in the chamber—usually in the evening—and were left there with intermittent observation for about 16 hours. Healthy adult cats were used which had been acclimatized to the laboratory environment at least four months. A few more female than male cats were used, but an equal sex distribution was achieved between the fasted and fed groups.

After the first three experiments the respiratory rate and estimated degree of salivation were recorded at each observation. Respiratory rates were counted in triplicate by gross inspection with a stop watch.

A suspension of dried brewer's yeast (Vita Food Green Label), 20 per cent by weight in skim milk, plus 5 per cent liver powder (Lilly) was given by gastric intubation in the first three experiments, whereas a 25 per cent suspension of yeast in skim milk was administered subsequently. The fasted animals were offered no food after their last regular meal, either 24 or 48 hours prior to exposure, and in the first three experiments were given water by intubation in amounts equal to the yeast-milk feedings.

In a first series of three experiments, it was planned to determine the effect of high protein intake and of p-aminobenzoic acid (PAB), a possible antidote (4), on survival, pathologic changes, methemoglobinemia, and Heinz body formation after exposure to xylidine vapors. Methemoglobin (MHb) and total hemoglobin concentration were determined according to the method of Horecker and Brackett (5). The blood turbidity ratio, which is

a quantitative photometric measure of the turbidity imparted to hemolyzed blood by the insoluble Heinz bodies, was measured by the method of Horecker (6) The force fed animals were given 20 cc per kgm of yeast liver milk suspension three times during the 8 hours preceding exposure in all three experiments, and were given the same amount once, 24 hours before exposure in the first two experiments or tests The xylidine concentration determined once during the exposure was 0 18 and 0 17 mgm per liter in the first and second tests

A second series of 8 experiments was carried out employing the same exposure procedure as previously except on two tests which were started in midforenoon The yeast milk suspension was tube fed in 75 (± 25) cc amounts once daily, 1 to 4 days before exposure and in 60 (± 10) cc amounts once or twice in the 6 hours preceding the start of exposure The xylidine concentration measured at different times on several runs varied between 0 11 and 0 27 mgm per liter

In a final series of 5 experiments the fed animals were given by intubation about 55 cc of yeast milk mixture or skim milk 2 to 4 hours and 50 cc one half to one hour before being exposed One of the two fed animals in each of the last two tests received skim milk without yeast In addition, the non fasted cats were offered their regular diet of meat and milk after the first intubation The xylidine concentration in the chamber atmosphere measured once during each test was 0 10 ± 035 mgm per liter

RESULTS As noted in table I, all the fasted animals survived more than 24 hours after exposure, whereas 29 per cent of the fed animals died during exposure, and 17 per cent died the following day Inasmuch as the effect of feeding on xylidine toxicity appeared to be an acute one, only the early survival data are given Serious hepatic and other lesions developed later, equalizing the overall survival in the two groups

The mean of the respiratory rates for all cats observed at specific intervals during, and in some instances before, exposure are shown in table II The observation time varied in different tests, so the number of cats on which the mean was determined differs at each interval The wide range of variation in the rates might be in part attributed to the tendency—referred to later—for the toxicity of xylidine, and the effect of feeding on this toxicity, to vary with the season

A rate of 70 or more per minute was observed in 94 per cent of the fed animals and in only 22 per cent of the fasted cats A rate of 200 or more per minute occurred in 59 per cent of the fed group but in only 4 per cent of the fasted animals A respiratory rate greater than 200 was observed in 80 per cent of the animals in the second and third series that died within 24 hours of exposure The accelerated respiration first appeared from 15 minutes to several hours after onset of exposure and generally wore off toward the end of exposure The rate often decreased markedly shortly after an emesis

The one animal exposed first in the fasted and months later in the fed condition developed tachypnea only on the latter occasion Many of the fed animals, but none of the fasted, developed a syndrome characterized by marked agitation, panting, cyanosis, ptyalorrhea and a glassy fixed expression about the eyes

It was evident that the degree of ptyalism resulting from the xylidine inhalation was greater in the fed animals No salivation was noted in 74 per cent of the fasted animals in the second and third series, and only a moderate degree of ptyalism was observed in the remaining 26 per cent This contrasts with the 55

per cent of the fed animals showing marked to extreme ptyalism in which a steady flow of saliva dripped from the mouth and soaked the face, thorax, and forelegs.

TABLE I

Survival of fed and fasted cats exposed to xylidine inhalation

EXPERIMENTS	TOTAL NO. CATS	NO. DIED DURING EXPOSURE	NO. DIED WITHIN 24 HRS. AFTER END OF EXPOSURE	NO. SURVIVED > 24 HRS.
		Fasted animals		
1– 3*	12	—	—	12
4–11†	17	—	—	14
12–15	10	—	—	10
		Fed animals		
1– 3	6	5	1	0
4–11†	17	4	—	10
12–16‡	15	2	5	8

* Half the animals received PAB solution instead of water at intubation.

† A fasted and fed animal sacrificed for histopathology at end of exposure in three experiments.

‡ Survival the same in milk and milk + yeast-fed groups.

TABLE II

Respiratory rates in fed and fasted cats before and during xylidine exposure (Respirations per minute)

HR. FROM START OF EXPOSURE	FASTED ANIMALS			FED ANIMALS		
	No. of cats observed*	Mean respiratory rate	Std. dev.	No. of cats observed*	Mean respiratory rate	Std. dev.
			Before exposure			
3– 6	8	43.0	16.5	8	41.4	16.1
0– 2	8	40.3	15.7	14	44.6	17.0
			During exposure			
¼	8	28.6	11.5	9	40.1	12.9
1– 2	7	31.3	14.0	12	157.5	72.5
3– 4	27	45.6	31.3	29	186.8	96.7
5– 6	11	53.3	60.0	13	151.1	69.2
7– 9	10	28.7	9.4	15	133.0	95.5
13–16	6	24.2	9.5	9	57.8	49.8

* When rates were recorded twice in a given time interval for an animal, the average of these two was used in computing the mean for all the cats.

MHb and Heinz body determinations on most of the animals exposed in the second series of experiments failed to reveal a difference between the fasted and the fed groups at the end of exposure. That the hyperpnea in fed cats was

not due to a high level of methemoglobinemia was shown by the virtual absence of MHb from blood samples of three fed animals taken at a time when they exhibited marked tachypnea. The effect of the administration of PAB to half the fasted animals in the first series of experiments which tended to show that PAB enhanced and prolonged the xylidine-induced rise in the MHb and Heinz body levels will be reported elsewhere.

Certain incidental observations may be mentioned. Periodic erythrocyte counts and hemoglobin and hematocrit determinations revealed severe anemia in a few cats about 7 days after exposure, followed in the survivors by a marked reticulocytosis sometimes reaching 70 per cent. In addition, numerous large Heinz bodies and a pink benzidine-positive plasma were observed in the anemic cats. Animals weak and prostrate in the cage two or three days after exposure were seen on occasion to produce a red-orange discoloration of the sawdust where contaminated with saliva. Reddening of wood shavings—a commonly observed property of urine from animals treated with aniline—indicates in this instance excretion of xylidine metabolic derivatives in the saliva.

The first and third series of experiments carried out in succeeding summers showed the difference between fasted and fed animals more strikingly than did the second series of 8 tests carried out during the intervening winter. However, the average xylidine concentration attained in the first and third series fell respectively above and below that found in the intervening (winter) series. This indicates a seasonal effect, the causes for which have not been investigated.

PATHOLOGY. Studies were made on formalin-fixed tissues of 27 fasted and 26 force-fed cats exposed to xylidine. Sections were stained with azure eosinate and frequently for hemosiderin, fat (7) and hemoglobin (8). Pathologic changes varied with the length of survival.

Changes in the Liver. In the first 48 hours, there was usually centrolobular congestion. Most of the liver cells, centrolobularly or diffusely, showed increased cytoplasmic eosinophilia and a variable amount of fat; a few cells were necrotic. Cats dying 3 to 11 days after exposure (3 to 11-day cats) often showed extensive centrolobular necrosis with disappearance of many liver cells, leaving a collapsed congested stroma moderately infiltrated chiefly by hemosiderin-laden and fat-laden phagocytes. Proliferation of small bile ducts and of bile duct epithelium was common. Most of these cats were jaundiced and showed inspissated bile casts in many canaliculi and bile-stained material in some bile ducts. One jaundiced cat dying 53 days after exposure showed in addition to such changes some interportal fibrous bands suggesting a possible beginning cirrhosis. Four other cats examined after the eleventh day showed no significant changes.

Changes in Other Organs. Pulmonary edema of variable extent was noted in 10 of 16 one- to five-day cats that had a maximum respiratory rate of 150 or more per minute during exposure and in only 1 (rate 84) of 11 such cats with a maximum respiratory rate below 150 (24 to 144). It was not seen after the fifth day. The myocardium of cats dying within 4 days after exposure showed slight to moderately marked, patchy or diffuse, fatty degeneration. The kidneys were often congested, and some showed hyaline and granular casts or bile-

stained material in a few tubules. Hemoglobin casts were noted in seven of nine 5-day and 6-day cats. The thymus of cats dying within 4 days after exposure was often markedly congested; about half showed scattered petechiae. No petechiae were noted after the fourth day, but involution of the thymus with cortical depletion of lymphocytes was common. The adrenal cortex of one 3-day cat showed several areas of hemorrhagic necrosis. The spleen of one 5-day cat showed several infarcts. Hemosiderosis of the spleen, liver, lungs and renal convoluted tubules was frequently marked after the third day.

DISCUSSION. Pulmonary edema noted here for the first time as a feature of xylidine toxicity was confined to, and involved the majority of, 1- to 5-day cats showing marked hyperpnea. Whether the edema constitutes a basis for the hyperpnea or simply occurs with it as a result of the same or separate etiologic processes remains a question. There is experimental evidence in rats that petechiae in the thymus may occur after severe anoxia (9) and rapid involution of the thymus may occur after a variety of noxious agents (10). Perhaps this may explain the changes in the thymus of our exposed cats.

It is thought that the severe anemia noted in a few cats was due to intravascular hemolysis since in these animals there was observed a pink, benzidine-positive plasma. Moreover, cats dying after the fourth day often showed hemoglobin casts in the renal tubules, brown benzidine-positive sediment in the urinary bladder, and hemosiderosis of the viscera.

The greater susceptibility of fed cats to acute effects of xylidine vapors cannot be attributed to a summation of toxic effects, for the milk, or yeast and milk feedings alone, produced no significant symptoms or lesions. Emesis occurring during exposure was often followed by considerable relief of respiratory distress. Symptoms in the fed animals subsided after exposure for several hours, so that the survivors in this group, aside from appearing weaker, resembled the controls at the end of exposure. The enteric absorptive process is apparently required for development of the effect.

As an explanation for the increased susceptibility during food absorption, it may be postulated that the postprandial acceleration in metabolism of amino acids—some of them aromatic amines—opens pathways whereby the oxidation, conjugation, or other metabolic alteration of xylidine to toxic intermediaries is augmented. Perhaps, also, the increased splanchnic blood and lymph flow in fed animals augments reabsorption of toxic derivatives excreted in the intestine (3). Finally, it is possible the increased circulatory and metabolic load in the postabsorptive state may overburden a toxic heart which is probably functionally impaired, as suggested by the fatty degeneration noted above.

SUMMARY

In contrast to fasted cats, those fed milk or milk and yeast reacted to xylidine vapors with an acute disturbance characterized by hyperpnea, panting, ptyalism, and frequently early death. Survivors of both groups developed a high level of MHb and Heinz bodies in the blood, often followed by a severe hemolytic anemia and jaundice.

Pathologic studies showed pulmonary edema occurring chiefly in the hyperpneic animals and lesions of the liver, kidney, heart and thymus occurring in both groups

REFERENCES

1 Heubner, W, and Schwedtke, G Arch f Exp Path u Pharmacol, 184 80, 1937
2 Scheff, G J This Journal, 70 334, 1940
3 Von Oettingen, W F, et al Nat Inst Health Bull, 188, 1945
4 Sandground, J H This Journal, 78 109, 1943
5 Horecker, B L, and Brackett, F S J Biol Chem, 152 669, 1943
6 Horecker, B L J Indust Hyg and Toxicol, 27 85, 1945
7 Lillie, R D Histopathologic Technic The Blakiston Company, Philadelphia, 1948
8 Dunn, R C, and Thompson, E C Arch Path, 39 49, 1945
9 Dalton, A J, Jones, B F, Peters, V B, and Mitchell, E R J Nat Cancer Inst, 6 161, 1945
10 Selye, H Brit J Exp Path, 17: 234, 1936

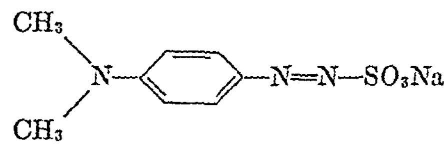
STUDIES ON THE TOXICITY AND PHARMACOLOGICAL ACTION OF p-DIMETHYLAMINOBENZENEDIAZO SODIUM SULFONATE (DAS)

ROY G. HERRMANN AND KENNETH P. DuBOIS

University of Chicago Toxicity Laboratory[1] *and the Department of Pharmacology, University of Chicago, Chicago, Illinois*

Recieved for publication November 3, 1948

Among the interesting chemical compounds which came to the attention of the Technical Intelligence Committee at the end of the recent war in Germany was p-dimethylaminobenzenediazo sodium sulfonate (DAS) which has the following chemical structure:



This compound has been employed in Germany as a rodenticide; however, information concerning the toxicity of DAS, its efficacy as a rodenticide and its pharmacological action has been lacking in this country. Our interest in this new compound was stimulated not only by its possible value as a rodenticide but also by the close chemical similarity between DAS and the carcinogenic azo dyes.

The present investigation on DAS was carried out to (a) measure the acute toxicity to several species (b) ascertain its efficacy as a rodenticide and (c) observe the pathological and pharmacological effects produced by the compound. Some experiments were also performed on N,N-dimethyl-p-phenylenediamine since this compound was the starting product for the synthesis of DAS and might be formed during the metabolism of DAS as it is in the case of other similar azo dyes (1). Comparable experiments with DAS and N,N-dimethyl-p-phenylenediamine might, therefore, indicate whether the parent compound or a diamine derivative was responsible for the toxic action.

METHODS. Sprague-Dawley rats (*ca.* 200 grams), Carworth mice (*ca.* 20 grams), adult guinea pigs (*ca.* 600 grams), adult rabbits (*ca.* 3 kgm.) and adult dogs were employed for these studies. DAS was synthesized by diazotization of N,N-dimethyl-p-phenylenediamine and subsequent sulfonation according to the procedure of Stollé (2). Aqueous solutions of DAS and N,N-dimethyl-p-phenylenediamine were administered intraperitoneally and mortality was observed for 10 days following administration of the drug.

For blood studies on rats and guinea pigs samples were taken by cardiac puncture from ether-anesthetized animals before and at intervals after the administration of DAS or the

[1] The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

diamine. Blood samples were taken from the marginal ear vein of unanesthetized rabbits and by cardiac puncture from mice under sodium pentobarbital anesthesia (80 mgm./kgm. intraperitoneally). Successive blood samples were taken from the same animal except in the case of mice where only one sample of blood was withdrawn from each mouse.

Blood glucose was measured by the method of Folin and Malmros (3) using a Somogyi blood filtrate (4) and glycogen was determined according to the method of Good *et al.* (5). For the measurement of the reducing value of urine the samples of urine were diluted with an equal volume of 0.05 *N* oxalic acid, then thoroughly mixed with Fuller's Earth (150 mgm./cc.) and centrifuged (6). A Somogyi filtrate was prepared from the supernatant liquid and the reducing value was measured by the same procedure employed for the blood samples.

EXPERIMENTAL. *Toxicity of DAS and N,N-dimethyl-p-phenylenediamine to mammals.* The acute toxicity of DAS to mammals was measured in order to evaluate the rodenticidal action of the compound and to ascertain which species exhibited the greatest susceptibility toward the toxic agent. Comparable toxicity measurements were performed on N,N-dimethyl-p-phenylenediamine to observe whether the species susceptibility to this compound was similar to that for DAS since such a comparison might indicate whether the acute toxic action of DAS was due to the parent molecule or a diamine resulting from the degradation of DAS *in vivo* at the azo linkage. The acute toxicity of DAS and N,N-dimethyl-p-phenylenediamine given intraperitoneally to five species is shown by the data in table 1. The approximate LD_{50} values for rats, mice and guinea pigs were determined by the log probability method while values for rabbits and dogs were obtained by gross inspection of the data.

These toxicity data demonstrate that there is considerable variation in the susceptibility of different species to DAS. In comparison with rats for which the LD_{50} was 15 mgm./kgm., mice were over four times more resistant, guinea pigs twice as resistant and dogs and rabbits exhibited a susceptibility similar to that shown by rats. No sex or age differences in the susceptibility of animals to DAS were observed.

The species variation in susceptibility to N,N-dimethyl-p-phenylenediamine differed considerably from that observed with DAS. The LD_{50} for mice and rats was nearly the same, being 21 and 25 mgm./kgm., respectively, while rabbits were about five times more resistant and guinea pigs twice as resistant as mice toward the compound. Thus, the difference in susceptibility of various species to the two compounds suggests that the toxic action of DAS does not depend upon its conversion to a diamine *in vivo*.

Symptomatology and pharmacodynamic observations of DAS. Acutely toxic doses of DAS produced a general depression and an apparent exhaustion of energy in all of the species studied. In rats after twice the LD_{50} dose these reactions began within 2 hours after poisoning with the depression progressing until, in the terminal stages of poisoning, the animals became flaccid and made no attempt to right themselves when placed on their backs. Sometimes slight tremors were observed and convulsions almost always preceded death. Ordinarily death occurred within ten hours after acutely lethal doses of DAS but sometimes it was delayed for several days.

In dogs anesthetized with sodium pentobarbital (35 mgm./kgm. intraperitoneally) the blood pressure, as measured with a mercury manometer connected to a cannulated carotid artery, decreased after the intravenous administration of 30 mgm./kgm. of DAS. Five minutes after the injection of DAS the blood pressure began to decrease and by 35 minutes it had fallen to the extent of 45 mm. of mercury with no further decrease until death of the animals occurred. About 0.5 hour after DAS was administered the depth of respiration decreased, and the rate increased from 19 to 24/minute. The depth of respiration then increased and remained above normal for about 3 hours while the rate remained around 22/minute for 2 hours then gradually decreased. At 3 hours after DAS was administered the respiration became shallow and irregular and ceased in about 15 minutes at which time artificial respiration was begun. With continuous artificial respiration the blood pressure gradually dropped and the heart ceased beating about 20 minutes after the cessation of respiration.

TABLE 1

Toxicity of DAS and N,N-Dimethyl-p-phenylenediamine given intraperitoneally to mammals

SPECIES	DAS		N,N-DIMETHYL-p-PHENYLENEDIAMINE	
	No. of animals used	LD_{50}	No. of animals used	LD_{50}
		mgm./kgm.		*mgm./kgm.*
Rats	74	15	47	21
Mice	70	70	89	25
Guinea pigs	36	30	32	45
Rabbits	12	10–20	8	100
Dogs	5	5–10	4	10–20

The symptoms which followed acute poisoning by N,N-dimethyl-p-phenylenediamine differed markedly from those produced by DAS. Within 10 to 30 minutes after the intraperitoneal administration of the compound animals developed tonic and clonic convulsions. Preceding the convulsions there existed a state of hyperexcitability which was soon followed by a series of convulsive seizures which terminated with death in about one hour after administration of the compound.

Pathological findings in rats after acute poisoning by DAS. As early as 15 to 30 minutes after the intraperitoneal administration of lethal doses of DAS to rats the yellow color of the compound was seen in the skin of the animals and was especially visible in the ears. The color was also observed in the urine as soon as 5 to 10 minutes after poisoning.

The gross pathological findings in rats acutely poisoned by DAS were not pronounced. Ordinarily the liver showed evidence of hyperemia of varying degrees of severity and pulmonary congestion was occasionally observed but pleural effusion was rarely noted. The kidneys of animals surviving for more than two days before succumbing to the poison were pale and swollen.

Microscopically the main pathological finding in rats 5 hours after the administration of 30 mgm./kgm. of DAS was a degeneration of the kidney tubules. Acute passive hyperemia of the liver was another consistent finding and occasionally a mild hyperemia could be detected in the mucosa of the gastrointestinal tract.

Acceptability of DAS to rats. To ascertain whether DAS was acceptable when offered to rats in the diet, feeding experiments were conducted. Diets containing 0.25 per cent, 0.5 per cent, and 1.0 per cent DAS were prepared by mixing the rodenticide with finely ground Purina Laboratory Chow. The diets were offered to unstarved rats overnight in place of their ordinary Purina Chow diet. For the five animals receiving 1 per cent DAS the average food consumption during the 12-hour period was 1.7 grams of food/kgm. and 4 of the 5 animals succumbed. When 0.5 per cent DAS was offered to 5 rats overnight the average food consumption was 4.9 grams/200-gram rat and all of the five animals died, and when 0.25 per cent DAS was offered the average food consumption was 4.9 grams/200-gram rat and three of the five animals died. These experiments indicated that DAS was effective and acceptable to rats when placed in the diet.

Because of the chemical similarity between DAS and the carcinogenic azo dyes, rats were fed ground Purina Laboratory Chow containing 0.10 per cent DAS to observe whether hepatomas would result. In animals which survived the feeding period hepatomas resembling those produced by dimethylaminoazobenzene appeared in approximately 12 months after the animals were placed on the diet. It is possible that the carcinogenic action might have become evident in a shorter period if synthetic diets optimum for carcinogenesis by azo dyes had been used.

To observe whether rats develop tolerance toward DAS after the administration of sublethal doses of the rodenticide five rats were given 10 mgm./kgm. of DAS intraperitoneally followed by a second injection of 20 mgm./kgm. three days later. All of the animals were dead within five hours after the second injection. Since the LD_{50} of DAS to rats is around 15 mgm./kgm. the inability of the animals to withstand a second dose of 20 mgm./kgm. indicated that little tolerance could have resulted from the first dose of the rodenticide.

The effects of DAS on blood glucose. In order to observe whether DAS interfered with carbohydrate metabolism blood glucose measurements were made on rats acutely poisoned by the rodenticide. The terminal convulsions produced by lethal doses of DAS suggested the possibility of a disturbance in carbohydrate metabolism. Blood glucose measurements were made before and at various intervals after the administration of the rodenticide. Rats, mice, guinea pigs and rabbits were employed to ascertain whether any species difference in the effects of DAS on carbohydrate metabolism could be observed.

The results of these measurements are shown in table 2 in which each value represents the average for at least four animals unless otherwise indicated by the number in parenthesis. It may be seen from these data that DAS caused hyperglycemia in all of the species studied with the degree of hyperglycemia

depending upon the dose of the drug and the species susceptibility to the toxic agent. Rats showed a relatively mild degree of hyperglycemia after the administration of 15 mgm./kgm. of DAS (LD_{50}) whereas twice the LD_{50} dose produced a marked hyperglycemia (172 mgm. per cent) within three hours after poisoning. Seven hours after poisoning the blood glucose fell to hypoglycemic levels and at the time of death it reached a very low level coinciding with the appearance of the terminal convulsions. The administration of 60 mgm./kgm. of DAS to rats caused a very rapid onset of hyperglycemia with a precipitious fall to hypoglycemic levels. Mice and guinea pigs showed similar changes but required a higher dose of the rodenticide to produce these changes, which corresponds to their greater resistance toward DAS. Rabbits, however, were quite resistant to the blood glucose changes produced by DAS and required approximately four times the LD_{50} dose before hyperglycemia developed. Thus, DAS produced hyperglycemia in rats, rabbits, mice and rats with the extent and time of onset being dependent upon the dose of the drug and the species susceptibility to the rodenticide.

TABLE 2

Effect of DAS on the blood glucose of rats, mice, guinea pigs and rabbits

SPECIES	DOSE OF DAS	BLOOD GLUCOSE, MGM. PER CENT						
		Hours after poisoning						
		Normal	1	3	5	7	10	At death
	mgm./kgm.							
Rats	15	104	113	111	102	106		
	30	94	131	172	80	61		25
	60	99	205	62	45 (1)			
Mice	30	117			99			
	80	114						12
Guinea pigs	30	95	106	100	104			
	60	107	132	225	199		110	
Rabbits	30	90 (2)	91 (2)	97 (2)	97 (2)		86 (1)	
	60	82 (1)	227 (1)	dead				

We were interested in comparing the effects of N,N-dimethyl-p-phenylenediamine on blood glucose with those produced by DAS. For these tests rats were poisoned with 25 mgm./kgm. of the diamine and blood samples were taken before the administration of the poison, at the onset of convulsions, and immediately preceding death. The average normal blood glucose level was found to be 89 mgm. per cent. At the beginning of convulsions, in from 5 to 12 minutes after poisoning, the average value was 100 mgm. per cent and just preceding death, in from 35 to 44 minutes after poisoning, it had risen to 125 mgm. per cent.

These results demonstrated that the diamine produced a slight increase in blood sugar but much less than that observed in DAS-poisoned animals

Glycogen content of tissues from DAS poisoned animals The terminal hypoglycemia which was observed in DAS-poisoned animals suggested that the glycogen stores might be depleted This was tested by measuring the liver glycogen of normal and DAS-poisoned animals which had been fasted for comparable periods of time The results of glycogen measurements on rats, mice, and guinea pigs poisoned by DAS are shown in table 3 in which each value represents the average amount of glycogen for at least four animals

These data show that acute poisoning by DAS results in a marked decrease in liver glycogen in the three species employed and that the degree of depletion depended upon the dose of the drug administered to a given species There was also a correlation between the species susceptibility to DAS and the dose required to cause a fall in liver glycogen as evidenced by the observation that 30 mgm /kgm of DAS nearly depleted the liver glycogen of rats while the same

TABLE 3

The effect of DAS on liver glycogen of rats, mice and guinea pigs

SPECIES	DAS	HOURS FASTED	PER CENT GLYCOGEN	
			Normal	5 Hours after poisoning
	mgm /kgm			
Rats	15	5	4 56	3 12
	30	5	2 14	0 114
Mice	30	5	0 79	0 74
	80	5	0 36	0 034
Guinea pigs	30	20	0 296	0 143
	60	20	0 296	0 132

dose had no significant effect on the liver glycogen of mice Muscle glycogen of rats also decreased after DAS, a dose of 30 mgm /kgm of DAS reduced the skeletal muscle glycogen of rats to one half of the normal value These studies on glycogen are in accord with the observations made on the blood glucose in which the response depended upon the dose of DAS and the species susceptibility to the rodenticide They also give support to the possibility that the hypoglycemia is a result of the depletion of liver glycogen

Antagonistic action of insulin and adrenal-demedullation toward the hyperglycemic action of DAS To ascertain whether epinephrine was involved in the hyperglycemia and the depletion of liver glycogen by DAS, blood glucose and liver glycogen measurements were made on rats from which the adrenal medulla had been removed eleven days prior to use for the experiment Similar measurements were also made on DAS poisoned and normal rats treated with insulin Two units of insulin/kgm were injected subcutaneously one hour before the administration of DAS and a second injection was given three hours

rat/hour. These values indicate an increase in glucose and/or reducing substances in the urine after DAS poisoning but do not seem great enough to substantiate a marked decrease in tubular reabsorption of glucose during the sampling period.

Discussion. The present investigation has included a number of experiments designed to obtain information which would permit an evaluation of the rodenticidal action of p-dimethylaminobenzenediazo sodium sulfonate (DAS). Toxicity tests have shown that the LD_{50} for DAS given intraperitoneally lies between 10 and 30 mgm./kgm. for albino rats, guinea pigs, rabbits and dogs. Thus, there was a relatively small variation in the susceptibility of these species to the rodenticide. On the other hand, mice were found to be considerably more resistant toward the compound. The oral LD_{50} for albino rats was about 55 mgm./kgm. and it is, therefore, several times less toxic than sodium fluoroacetate (7) and alpha-naphthylthiourea (8). A marked difference in species susceptibility to DAS and N,N-dimethyl-p-phenylenediamine was observed which suggests that the compounds act differently and that the toxic action of DAS is not due to a diamine degradation product formed by cleavage at the azo linkage.

No significant age or sex difference in the susceptibility of rats to DAS was noted and the administration of a sublethal dose did not result in the acquisition of tolerance. The compound was acceptable to rats when offered at a concentration of 1 per cent or less in the diet. The main disadvantages attending the use of DAS as a rodenticide are its lower toxicity as compared with other new rodenticides and the absence of an effective antidote. Nevertheless, the substance may find practical use where the extremely high toxicity of fluoroacetate to many species (7) or the tolerance toward ANTU (8) limit the usefulness of these substances.

Because of the carcinogenic action of DAS the compound may be of value in future studies on the mechanism of carcinogenesis by azo dyes. Although it is a less potent carcinogen than several other azo dyes it presents an advantage in that it differs from the other carcinogenic azo dyes in being readily soluble in water and thus can be employed conveniently for *in vitro* experiments.

Studies on the blood glucose and tissue glycogen of DAS-poisoned animals have shown that this rodenticide produces a marked disturbance in carbohydrate metabolism. The hyperglycemia appeared to be induced by epinephrine since it could be prevented by adrenal-demedullation and insulin but these treatments did not affect the survival of DAS-poisoned animals. The terminal convulsions were due to hypoglycemia since they could be prevented or stopped by the administration of glucose. The hypoglycemia was undoubtedly the result of the depletion of tissue glycogen in animals poisoned by DAS, and the glycogen depletion seemed to be due principally to an inability of the tissues of DAS-poisoned rats to deposit glycogen. These results strongly suggest that DAS inhibits one or more of the enzymatic reactions involved in intermediary carbohydrate metabolism and future studies on the effects of DAS on enzyme systems may provide an explanation for the acute toxic action of the rodenticide.

SUMMARY

1 Measurement of the toxicity of p dimethylaminobenzenediazo sodium sulfonate (DAS) administered intraperitoneally gave the following approximate LD_{50} values in mgm /kgm albino rats 15, albino mice 70, guinea pigs 30, rabbits 10–20, and dogs 5–10 The oral LD_{50} for albino rats was about 55 mgm /kgm

2 No tolerance to DAS was observed in rats and the rodenticide was acceptable and produced high mortality of rats when placed in the diet at concentrations of 0 5 per cent and 1 0 per cent

3 Toxicity measurements on N,N dimethyl p phenylenediamine given intraperitoneally gave the following LD_{50} values in mgm /kgm albino rats 21, mice 25, guinea pigs 45, rabbits 100, and dogs 10–20 The differences in species susceptibility to this compound and DAS suggest that the latter substance does not undergo cleavage at the azo linkage before exerting its toxic effect

4 DAS caused a generalized depression in all species, degeneration of the kidney tubules, hyperemia of the intestine and liver, a fall in blood pressure and respiratory paralysis

5 Hyperglycemia followed by hypoglycemia occurred in animals poisoned by DAS The extent of these changes was dependent upon the dose of DAS and the species susceptibility to the agent The hyperglycemia could be prevented by insulin or by adrenal demedullation but survival of the animals was not influenced by these treatments The hypoglycemic convulsions could be terminated or prevented by glucose which prolonged the survival time but did not prevent ultimate death of the animals

6 A depletion of liver and muscle glycogen of rats was observed after administration of DAS and the animals were unable to deposit liver glycogen from injected glucose, this indicated that DAS exerts an inhibitory action on glycogen synthesis

Acknowledgements The authors are indebted to Dr C S Hamilton, Professor of Chemistry, University of Nebraska, for valuable suggestions concerning the synthesis of p dimethylaminobenzenediazo sodium sulfonate and to Dr John W Green of the Department of Pathology, University of Chicago, for the microscopic examination of tissues from poisoned animals

REFERENCES

1 Stevenson, E S, Dobriner, K, and Rhoads, C P Cancer Res, 2 160, 1942
2 Stollé, R Berichte, 45 2682, 1912
3 Folin, O, and Malmros, H J Biol Chem, 83 115, 1929
4 Somogyi, M J Biol Chem, 86 655, 1930
5 Good, C A, Kramer, H, and Somogyi M J Biol Chem, 100 484, 1933
6 Koch, F C, and Hanke M E Practical Methods in Biochemistry 4th Ed, Williams & Wilkins, 246, 1943
7 Kalmbach, E R Science, 102 232, 1945
8 Richter, C P J A M A, 129 927, 1945

THE EFFECT OF p-DIMETHYLAMINOBENZENEDIAZO SODIUM SULFONATE (DAS) ON THE ENZYMATIC REACTIONS OF INTERMEDIARY CARBOHYDRATE METABOLISM

ROY G. HERRMANN AND KENNETH P. DuBOIS

University of Chicago Toxicity Laboratory[1] *and the Department of Pharmacology, University of Chicago, Chicago, Illinois*

Received for publication November 3, 1948

Evidence that acute poisoning by p-dimethylaminobenzenediazo sodium sulfonate (DAS) results in a disturbance of carbohydrate metabolism was obtained during an investigation of the toxicity and pharmacological action of this German rodenticide. This evidence, which is presented in the preceding communication, consisted of the observation that hyperglycemia followed by hypoglycemia and depletion of liver glycogen occurred after acute poisoning by DAS. The inability of fasted DAS-poisoned rats to deposit liver glycogen from injected glucose indicated that the toxic agent exerted an inhibitory action on glycogen synthesis. These findings stimulated our interest in investigating the action of DAS on the enzymatic reactions involved in intermediary carbohydrate metabolism in an effort to elucidate the mechanism of the acute toxic action of this azo compound in mammals.

The present communication contains the results of tests on the effect of DAS on several enzymatic reactions. In this study attempts were made to correlate *in vitro* findings with *in vivo* results in which the enzymatic reactions of tissues from DAS-poisoned animals were studied. Such attempted correlations are of considerable importance in explaining drug action on the basis of inhibition of enzymatic reactions. The results obtained in this study have demonstrated that DAS inhibits the oxygen consumption of liver slices and exerts a potent inhibitory action on the aerobic synthesis of high-energy phosphate compounds *in vitro* and *in vivo*. The inhibitory action of DAS on aerobic phosphorylation was dependent upon the dose of the rodenticide and upon the species susceptibility to the toxic compound.

METHODS. Sprague-Dawley rats (ca. 200 grams), Carworth mice (ca. 20 grams) and adult guinea pigs (ca. 600 grams) were employed for these studies. DAS was synthesized according to the procedure of Stollé (1) and aqueous solutions of the compound were administered by the intraperitoneal route.

Anaerobic glycolysis of brain tissue was measured according to the method of Elliott and Henry (2) and the oxidation of glucose by brain homogenates was measured by the procedure of Elliott *et al.* (3). The oxidation of several substrates by liver slices was measured using rat liver slices prepared according to the method of Deutsch (4). The liver slices were obtained from normal rats fasted for 16 hours prior to being sacrificed. Succinic de-

[1] The work described in this paper was done under contract between the Medical Division, Chemical Corps, U. S. Army and the University of Chicago Toxicity Laboratory. Under the terms of the contract the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

hydrogenase and cytochrome oxidase activities were determined by the method of Schneider and Potter (5), malic dehydrogenase by the method of Potter (6) and adenosine triphosphatase by the procedure of DuBois and Potter (7).

The extraction, fractionation and measurement of the acid-soluble phosphorus compounds of rat tissues were carried out according to the procedure outlined by LePage and Umbreit (8). For these measurements the tissues were rapidly removed during sodium pentobarbital anesthesia (45 mgm./kgm.), and quickly frozen between two blocks of dry ice.

The effect of DAS on oxidative phosphorylation was ascertained by the method of Potter (9) using 0.3 cc. of a 10 per cent isotonic potassium chloride homogenate as the source of the enzymes, 26 mgm. of anhydrous creatine as the phosphate acceptor and succinate as the oxidizable substrate.

EXPERIMENTAL. *The action of DAS on the succinoxidase system.* The possibility that DAS might be metabolized *in vivo* to yield p-phenylenediamine or a methyl derivative of p-phenylenediamine, which compounds are known to be strong inhibitors of succinic dehydrogenase (10), stimulated our interest in investigating the effect of DAS on succinic dehydrogenase. *In vitro* and *in vivo* measurements of succinic dehydrogenase activity were, therefore, carried out on rat liver, kidney, lung and brain tissues. Various concentrations of DAS were tested by adding all of the components of the reaction mixture together with the inhibitor to the main compartment of the Warburg vessel and incubating for 20 minutes before the addition of the succinate from the side arm.

The effect of DAS on the succinoxidase activity of rat liver is shown in figure 1 from which it may be seen that DAS had an inhibitory action on this enzyme system *in vitro* but in all cases there was a lag period of about 20 minutes after the addition of the succinate before the inhibitory action of DAS became apparent. This was interpreted as indicating that some chemical change in the toxic agent took place to yield a product which inhibited the enzyme. It was noticed that the inhibition occurred after most of the yellow color due to DAS had disappeared from the reaction mixture which further suggested that a change in the DAS molecule occurred. As shown by Curves B and C of figure 1 final concentrations of $6.6 \times 10^{-5}\,M$ and $1 \times 10^{-4}\,M$ DAS respectively produced marked inhibition of enzyme activity when the inhibitor was incubated with the enzyme for 20 minutes before the addition of the substrate. Placing the inhibitor in the side-arm of the Warburg vessel with the succinate and adding both simultaneously decreased the amount of inhibition (Curve D, figure 1) as compared with the addition of the substrate after the inhibitor. This indicated a competition between succinate and the rodenticide for the enzyme. In contrast to the *in vitro* results no decrease in the succinoxidase activity of liver was observed when the livers were removed 5 hours after the administration of 30 mgm./kgm. of DAS.

DAS also produced an inhibition of the succinic dehydrogenase activity of kidney tissue *in vitro* after a preliminary lag period similar to that observed with liver. A final concentration of $1 \times 10^{-4}\,M$ DAS produced 2 per cent inhibition at 30 minutes, 24 per cent inhibition at 70 minutes and 67 per cent inhibition at

110 minutes after the addition of the succinate. As in the case of liver, however, no significant inhibition of the succinoxidase activity of kidney was observed 5 hours after the administration of 30 mgm./kgm. of DAS. A final concentration of 1×10^{-4} *M* DAS produced no significant inhibition of the succinic dehydrogenase activity of brain and lung tissue *in vitro* nor was there any decrease in the activity of the enzyme in these tissues 5 hours after the administration of 30 mgm./kgm. of DAS. From these experiments it appeared that liver and kidney tissues are able to produce some change in DAS which results in the production of a succinic dehydrogenase inhibitor. In view of the inhibitory action of several phenylenediamines on succinic dehydrogenase which was observed by Potter and DuBois (10) it seems possible that the inhibitory

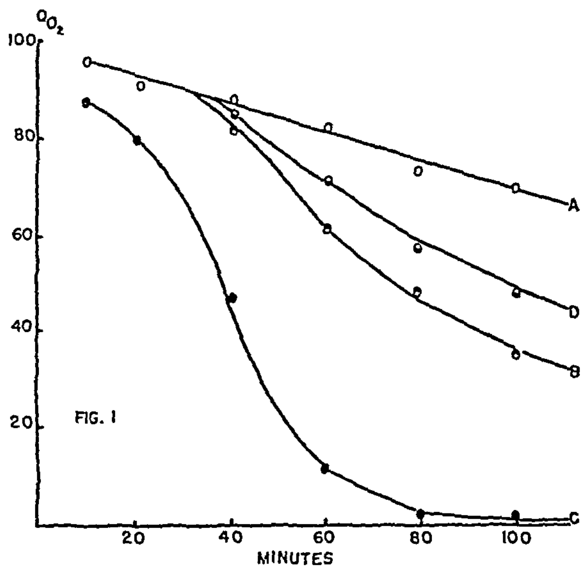


FIG. 1. THE EFFECT OF DAS ON THE SUCCINIC DEHYDROGENASE ACTIVITY OF RAT LIVER
Curve A, normal control; Curve B, 6.6×10^{-5} *M* DAS added to the enzyme 20 minutes before the addition of the succinate; Curve C, 1×10^{-4} *M* DAS added to the enzyme 20 minutes before the addition of the succinate; Curve D, 1×10^{-4} *M* DAS added to the enzyme together with the succinate.

action of DAS on this enzyme system was due to cleavage of DAS at the azo linkage to form a toxic diamine. The lack of an inhibitory action of DAS on succinic dehydrogenase activity *in vivo* suggested that some other enzyme was more sensitive toward the inhibitor or that the enzyme-inhibitor complex was dissociated during homogenization and dilution of the tissue.

Because of the possibility that N,N-dimethyl-p-phenylenediamine might occur as a breakdown product of DAS the effect of this compound on the succinic dehydrogenase activity of rat liver was tested. Four rats were given 25 mgm./kgm. of the diamine intraperitoneally and sacrificed during convulsions which occurred one-half to three-fourths of an hour after poisoning. Whereas the QO_2 for the succinic dehydrogenase activity of normal livers was 82 that for

the livers of poisoned animals was 79. This compound had previously been shown (10) to produce 77 per cent inhibition of succinic dehydrogenase at a final concentration of 1×10^{-5} *M in vitro*. DAS and N,N-dimethyl-p-phenylenediamine therefore act similarly on succinic dehydrogenase inhibiting the action of the enzyme *in vitro* but not *in vivo*.

Since the succinoxidase system (5) employed for these experiments requires the catalytic action of succinic dehydrogenase, cytochrome *c*, and cytochrome oxidase it was necessary to investigate the action of the toxic substance on the latter two catalysts before attributing its inhibitory effect solely to an interaction with succinic dehydrogenase. It was found that a final concentration of 2×10^{-4} *M* DAS had no effect on cytochrome oxidase activity of rat liver while the same concentration had produced complete inhibition of the succinoxidase system. Variation in the cytochrome *c* content of the succinoxidase system did not alter the per cent inhibition by DAS; therefore, DAS was not reacting with this component of the system and its inhibitory effect could be attributed to an action on succinic dehydrogenase.

The absence of an inhibitory effect by DAS on the malic dehydrogenase system. To test the effect of DAS on a coenzyme I-linked dehydrogenase the malic dehydrogenase system was employed. Whereas the normal QO_2 value for malic dehydrogenase of liver was 54 the addition of DAS at final molar concentrations of 1×10^{-3} and 1×10^{-4} resulted in QO_2 values of 48 and 53, respectively. DAS, therefore, produced no significant inhibition of malic dehydrogenase *in vitro*.

Using the malic dehydrogenase test system with fumarate in place of malate as the substrate it was possible to obtain an indication of whether DAS inhibits fumarase activity. The use of fumarate in place of malate as the substrate requires the catalytic action of fumarase to convert fumarate to malate which is then oxidized by the malic dehydrogenase system. The absence of an inhibitory effect by DAS at 1×10^{-3} *M* final concentration indicated that the rodenticide did not inhibit the activity of fumarase.

The influence of DAS on anaerobic glycolysis and the oxidation of glucose by brain and liver. In initiating studies on the effect of DAS on glycolysis and respiration isotonic brain homogenates and liver slices were employed. An inhibitory action by DAS on the overall processes of either glycolysis or respiration could then be followed by an examination of the action of the toxic compound on individual enzymatic reactions in an attempt to locate the specific site of action.

DAS exerted no effect on the rate of anaerobic glycolysis of rat brain homogenates and liver slices. In the case of brain the normal CO_2/N_2 was 8.2 and in the presence of final concentrations of 1×10^{-2} *M* and 1×10^{-3}*M* DAS the values were 8.0 and 7.5, respectively. Similarly, DAS had no effect on the rate of anaerobic glycolysis of liver slices. Whereas the normal QCO_2/N_2 value was 6.3 for rat liver slices the presence of a final concentration of 1×10^{-4} *M* DAS resulted in a value of 6.1. These experiments indicated that DAS in the concentrations employed had no significant effect on anaerobic glycolysis which seemed to eliminate inhibition of glycolytic enzymes from further consideration

in the search for the cause of the disturbance in carbohydrate metabolism produced by DAS.

The oxidation of glucose by rat brain homogenates was inhibited *in vitro* by DAS. Final concentrations of 1×10^{-3} *M*, 1×10^{-4} *M* and 1×10^{-5} *M* DAS produced 89, 63 and 40 per cent inhibition, respectively, of the oxidation of glucose. However, no depression of the rate of oxidation of glucose by brain tissue of rats given 30 mgm./kgm. of DAS could be detected when the animals were sacrificed 5 hours after administration of the compound.

DAS also inhibited the oxygen consumption of liver slices suspended in Krebs-Ringer phosphate solution (11) buffered at pH 7.4 and containing 0.02 *M* glucose. The presence of a final concentration of 1×10^{-4} *M* DAS depressed the QO_2 from a normal value of 7.0 to 3.8. An inhibitory action by DAS on the cellular respiration of liver slices *in vivo* also occurred. Livers removed from rats 5 hours after the administration of 30 mgm./kgm. of DAS gave an average QO_2 value 5.6 representing a 21 per cent decrease in oxygen consumption. The inhibitory action of DAS on cellular respiration suggested that further experiments should be directed toward studying the action of the rodenticide on the various steps of the oxidative phase of carbohydrate metabolism.

The inhibitory action of DAS on the oxidation of pyruvate and citrate by rat liver slices. The lack of an inhibitory action by DAS on the oxidation of succinate, malate and fumarate indicated that the depressant action of the toxic compound occurred at some other step in the oxidative phase of carbohydrate metabolism. The effect of DAS on the respiration of liver slices was therefore tested employing several of the intermediates of the Krebs citric acid cycle as substrates. The substrates employed for these experiments were fumarate, malate, succinate, citrate, pyruvate and oxalacetate. L-glutamate was also included in these studies.

The substrates were prepared as 0.3 *M* solutions with the exception of oxalacetate which was prepared as a 0.15 *M* solution. Two-tenths cc. of the substrate was added to enough Krebs-Ringer phosphate medium to give a final volume of 3.0 cc. in the Warburg vessel. Where DAS was tested *in vitro* 0.3 cc. of a solution (1×10^{-3} *M*) replaced an equivalent amount of Krebs-Ringer phosphate giving a final inhibitor concentration of 1×10^{-4} *M*.

For testing the effect of DAS on the oxidation of various substrates by liver slices one sample was included without the addition of a substrate to measure the endogenous respiration and another sample with an added substrate but without DAS was included to measure the stimulation of respiration due to the substrate. A third sample contained DAS and no added substrate to measure the effect of the compound on the endogenous respiration and another sample contained DAS and an added substrate to note the effect of DAS on the oxidation of the particular substrate tested. The results of these tests are shown in table 1 in which each value is the average of at least 3 determinations.

As may be seen from the data in table 1 the stimulation of the respiration of liver slices produced by malate, succinate and oxalacetate was unaffected by the addition of DAS. However, the additional oxygen consumption due to fumarate, pyruvate and citrate was decreased by the presence of 1×10^{-4} *M* DAS.

To ascertain whether the inhibition of the utilization of these oxidative substrates also occurred in DAS poisoned animals *in vivo* tests were performed on the livers of rats poisoned 5 hours previously with 30 mgm./kgm. of DAS.

TABLE 1

Effect of DAS in vitro and in vivo on the oxidation of several substrates by rat liver slices

SUBSTRATE	Q_{O_2}		
	Control	1×10^{-4} M DAS	In vivo 5 hours after 30 mgm./kgm. DAS
Fumarate	9.3	4.9	8.1
No fumarate	7.0	4.2	5.7
Stimulation	2.3	0.7	2.4
Malate	8.0	4.9	7.3
No malate	7.0	3.9	5.6
Stimulation	1.0	1.0	1.7
Succinate	15.9	11.4	14.8
No succinate	7.1	3.0	5.7
Stimulation	8.8	8.4	9.1
Citrate	10.5	4.0	7.8
No citrate	7.2	3.3	5.4
Stimulation	3.3	0.7	2.4
Pyruvate	10.1	5.1	7.3
No pyruvate	6.8	3.8	5.6
Stimulation	3.3	1.3	1.7
Oxalacetate	9.2	6.7	
No oxalacetate	6.9	4.2	
Stimulation	2.3	2.5	
l Glutamate	8.4		7.7
No l glutamate	6.7		5.5
Stimulation	1.7		2.2

The results of these tests are also shown in table 1 where it may be seen that the stimulation of respiration by malate, succinate, glutamate and fumarate was not decreased when the livers of poisoned rats were used in place of the livers of normal animals. The additional oxygen consumption due to pyruvate and citrate, however, was depressed in the liver samples from poisoned animals.

These findings are in agreement with results obtained on the isolated succinoxidase and malic dehydrogenase systems in which DAS did not exert an inhibitory action *in vivo*. The present experiments indicate that the inhibitory action of DAS on the oxidative phase of carbohydrate metabolism might be explained on the basis of inhibition of the oxidation of citrate and pyruvate.

The influence of DAS on the concentration of acid-soluble phosphorus compounds of rat liver and kidney. Although DAS had no direct inhibitory action on glycolysis the depression of the cellular respiration by the compound might lead to changes in the concentration of some of the phosphorylated intermediates of glycolysis. The dependence of the synthesis of high-energy phosphate bonds upon oxidative reactions made the possibility of depletion of such compounds by DAS seem probable. The concentrations of acid-soluble phosphorus compounds

TABLE 2

The distribution of acid-soluble phosphorus compounds in the liver and kidney of normal and DAS-poisoned rats

COMPOUND	LIVER		KIDNEY	
	Normal	5 hours after 30 mgm./kgm. DAS	Normal	5 hours after 30 mgm./kgm DAS
	(Micromoles per 100 grams of wet tissue)			
Phosphocreatine	406	0	22	0
Adenosine triphosphate	74	96	0	0
Adenosine diphosphate	245	207	0	0
Adenylic acid	83	88	143	130
Glucose-1-phosphate	136	85	163	131
Glucose-6-phosphate	554	675	454	575
Fructose-6-phosphate	19	20	25	25
Fructose-1,6-diphosphate	67	20	30	24
Triose phosphate	18	30	0	0
Phosphopyruvate	15	37	54	73
Coenzymes	9	9	18	17
Inorganic phosphorus	235	806	872	885
Total phosphorus	2670	2840	3025	2425

in rat tissues were, therefore, measured after the administration of 30 mgm./kgm. of DAS given intraperitoneally. The liver was chosen for these studies because DAS had been shown to produce biochemical changes in this organ as evidenced by depletion of liver glycogen and inhibition of cellular respiration. Kidney tissue was also included in these measurements because of the evidence that a degeneration of the kidney tubules occurs in rats poisoned by DAS. Four normal unfasted male rats and 4 rats sacrificed 5 hours after poisoning were employed for the measurements.

The results of these determinations are summarized in table 2 in which each value is the average for 4 rats. The data indicate that DAS produces changes in the distribution of the acid-soluble compounds of both liver and kidney of rats. The most pronounced change was a depletion of phosphocreatine of both liver and kidney tissue. The decrease in phosphocreatine partly accounts for

the increase in inorganic phosphorus in the tissues of poisoned animals There was also a slight decrease in glucose-1-phosphate and a small increase in glucose-6-phosphate as well as small increases in phosphopyruvate and triose phosphate

In order to ascertain whether the phosphocreatine of tissues other than liver and kidney was depleted in DAS poisoned animals measurements were made on heart and skeletal muscle of rats taken 5 hours after the intraperitoneal administration of 30 mgm /kgm of DAS It was found that DAS had no effect on the phosphocreatine level of these tissues in contrast to the effects on liver and kidney phosphocreatine

The inhibitory effect of DAS on oxidative phosphorylation by rat, mouse and guinea pig homogenates The depletion of phosphocreatine in liver and kidney tissue from DAS poisoned rats suggested the possibility that the toxic agent inhibited the esterification of phosphate Attention was drawn to this possi-

TABLE 3

The effect of DAS on oxidative phosphorylation by rat, mouse and guinea pig kidney homogenates in vitro

SPECIES	MOLAR CONCENTRATION OF DAS	E (MICROGRAMS OF P ESTERIFIED PER 30 MGM WET TISSUE)	CMM O_2 CONSUMED PER 10 MINUTES	% INHIBITION
Rats	Control	136	39	—
	1×10^{-4}	53	36	61
	1×10^{-5}	75	41	45
	5×10^{-6}	83	39	39
	1×10^{-6}	137	58	0
Mice	Control	115	36	—
	1×10^{-4}	30	36	74
Guinea pigs	Control	148	34	—
	1×10^{-4}	104	40	30
	1×10^{-5}	144	44	2

bility not only by the depletion of phosphocreatine but also by the inhibitory action of DAS on cellular respiration The recent development of a method by Potter (9) for measuring coupled oxidation and phosphorylation has made it possible to obtain quantitative measurements of the effect of drugs *in vitro* and *in vivo* on this important synthetic reaction It was thus possible to ascertain the effect of DAS on phosphocreatine synthesis

For our measurements 0 3 cc of a 10 per cent kidney homogenate prepared in isotonic KCl was employed as the source of the enzymes, succinate as the oxidizable substrate and creatine as the phosphate acceptor *In vitro* measurements were carried out on kidney tissue of normal animals employing various concentrations of DAS and *in vivo* measurements were performed on kidney tissue taken from poisoned animals The effects of various concentrations of DAS on aerobic phosphorylation *in vitro* are shown in table 3 in which E represents the micrograms of inorganic phosphate esterified by 30 mgm of wet tissue in 20 minutes

and O_2 represents the cmm. of oxygen consumed by the tissue during the same period of time (9).

The results of these tests demonstrated that DAS is a strong inhibitor of aerobic phosphorylation *in vitro*. At the low concentration of 5×10^{-6} M 39 per cent inhibition of transphosphorylation by rat kidney homogenates was observed without a significant effect on oxygen consumption. This indicated that the transphorylating enzyme was inhibited rather than the oxidation of succinate; thus the rodenticide essentially uncoupled phosphorylation and oxidation. The reaction was also inhibited when guinea pig and mouse kidneys were used as the source of the enzyme. However, in spite of the marked difference in the susceptibility of rats, mice and guinea pigs toward the acute toxic action of

TABLE 4

The effect of DAS on oxidative phosphorylation by rat, mouse and guinea pig kidney tissue in vivo

SPECIES	DOSE OF DAS	HOURS AFTER ADMINISTRATION DAS	E (MICROGRAMS OF P ESTERIFIED PER 30 MGM. WET TISSUE)	CMM. O_2 CONSUMED PER 10 MINS.	% INHIBITION
	mgm./kgm.				
Rats	normal	—	136	39	—
	15	5	32	37	76
	30	1	71	39	48
	30	5	−75	34	100
Mice	normal	—	115	36	—
	30	5	114	40	0
	90	5	17	43	85
Guinea pigs	normal	—	148	34	—
	30	5	102	31	31
	60	5	80	32	46

DAS there was no marked difference in the amount of DAS necessary to inhibit phosphorylation by the kidney tissue from the three species *in vitro*.

To ascertain the effect of DAS on aerobic phosphorylation *in vivo* rats, mice and guinea pigs were given various doses of the compound and the kidneys were removed 5 hours later for aerobic phosphorylation measurements. The results of these tests are shown in table 4 in which each value is the average for at least 4 animals. It may be seen from these data that DAS exerted a marked inhibitory action on phosphocreatine synthesis *in vivo* with the extent of inhibition being dependent upon the dose of the compound administered. Furthermore, there was a correlation between the species susceptibility to DAS and the dose required to inhibit the transphosphorylation system *in vivo*. The LD_{50} values for rats, guinea pigs and mice are 15, 30, 70 mgm./kgm. of DAS, respectively. While 30 mgm./kgm. of DAS produced complete inhibition of aerobic phosphorylation by kidney tissue of rats, no inhibition of the reaction in the

kidneys of mice and only 31 per cent inhibition occurred in guinea pig kidneys after this dose of DAS.

In all of the tests performed it was observed that the phosphorylation of creatine was inhibited without inhibition of the oxidation of succinate. This was not unexpected since experiments described above on succinic dehydrogenase demonstrated that the inhibition of this enzyme by DAS occurred only after a lag period of about 40 minutes from the time of addition of the inhibitor to the enzyme and since the phosphorylation experiments were performed in 20 minutes inhibition of succinic dehydrogenase would not be manifested during this period. These experiments indicate that DAS produces its inhibitory effect on some enzyme or enzymes directly involved in the transphosphorylation process.

It has recently been reported by Loomis and Lipmann (11) that dinitrophenol reversibly uncouples phosphorylation from oxidation. It was of interest to observe whether the inhibitory action of DAS on oxidative phosphorylation was also reversible. For these experiments a 20 per cent kidney homogenate was

TABLE 5

The reversibility of the inhibitory action of DAS on aerobic phosphorylation

TREATMENT	E (MICROGRAMS OF P ESTERIFIED PER 60 MGM WET TISSUE)	CMM O_2 CONSUMED PER 10 MINS
Unwashed enzyme preparation		
Control	102	39
1×10^{-4} *M* DAS added	−7	48
Washed enzyme preparation		
Control	76	48
1×10^{-4} *M* DAS added before washing	78	47
1×10^{-4} *M* DAS added after washing	−18	61

prepared in isotonic potassium chloride and divided into 3 portions one of which served as the control and received no further treatment To a 3 cc. portion of the homogenate DAS was added to give a final concentration of 1×10^{-4} *M* and the mixture was allowed to stand for 30 minutes after which time it was centrifuged at approximately 5000 rpm in a refrigerated centrifuge for 30 minutes. The supernatant liquid was then discarded and the precipitate was well mixed with 3 cc. of isotonic potassium chloride and again centrifuged After 3 such washings the precipitate was taken up in 3 cc. of potassium chloride solution and employed for measurements of oxidative phosphorylation. Another portion of the kidney homogenate without the addition of DAS was carried through the same procedure and served as a control to indicate the amount of loss of the enzyme during the washing procedure. In no instance was the temperature of the homogenates allowed to exceed 5°C. The phosphorylative activities of the samples are shown in table 5 in which it may be seen that the inhibitory action of DAS was reversible under the conditions of this experiment. While the washing procedure resulted in 26 per cent loss of phosphorylative activity of the homogenate the addition of DAS before washing the enzyme resulted in no

further loss of activity. This demonstrated that the inhibitor could be removed by washing the enzyme. That 1×10^{-4} *M* DAS would have resulted in complete inhibition of phosphorylation by this enzyme preparation if it were not removed by washing was adequately demonstrated by complete inhibition of the reaction after addition of DAS to the washed and the untreated kidney homogenate.

DISCUSSION. An investigation of the effects of p-dimethylaminobenzenediazo sodium sulfonate (DAS) on intermediary carbohydrate metabolism has shown that the compound has no direct inhibitory action on glycolysis but does exert an inhibitory effect on the oxidative phase of carbohydrate metabolism. In attempts to ascertain the exact site of action of DAS on the oxidation of carbohydrate several *in vitro* and *in vivo* tests were performed. DAS inhibited succinic dehydrogenase *in vitro* but the inhibition occurred only after a lag period during which time it appeared that the compound had undergone a chemical change. It is likely that cleavage at the azo linkage occurred to yield a diamine which then inhibited succinic dehydrogenase. The absence of an inhibitory effect by DAS on succinic dehydrogenase *in vivo* and on the oxidation of succinate by liver slices indicated that this enzyme was not involved in the acute toxic action of this azo compound. Similarly cytochrome oxidase, malic dehydrogenase and fumarase were eliminated from further consideration in the mechanism of the toxic action of DAS. The observation that the oxidation of pyruvate and citrate by liver slices was inhibited by DAS *in vitro* and *in vivo* indicated that DAS inhibits one or more of the biocatalysts involved in the formation of alpha-ketoglutarate from pyruvate. The action of DAS on the oxidation of carbohydrate can be explained, at least partially, on the basis of inhibition of this portion of the dicarboxylic acid cycle. This inhibitory effect may also be partially responsible for the inhibition of glycogen synthesis by DAS.

We attached considerable significance to the depletion of phosphocreatine in some of the tissues of DAS-poisoned rats. Phosphocreatine is generally assumed to constitute a store of energy-rich phosphate which together with adenosine triphosphate functions in the transfer of chemical energy into energy available for the performance of tissue functions. Since DAS had no direct effect on glycolysis we reasoned that the depletion of phosphocreatine might result from an inhibition of the aerobic phosphorylation of creatine by DAS. It was found that DAS had a potent inhibitory effect on the aerobic synthesis of phosphocreatine. This effect, however, was not due to inhibition of oxygen consumption but rather to a direct action on the transphosphorylating mechanism since no depression of oxygen consumption occurred in the test system employed for phosphorylation. Thus, DAS, like dinitrophenol (12), uncoupled oxidation from phosphate esterification.

In connection with the mechanism of acute poisoning by DAS greater significance is attached to the inhibition of phosphorylation than to any of the other effects produced by the compound on the enzymes studied. DAS inhibited aerobic phosphorylation both *in vivo* and *in vitro* and there was a correlation between the species susceptibility to acute poisoning by DAS and the dose

of the compound necessary to inhibit aerobic phosphorylation *in vivo* *In vitro*, however, the kidney tissue of rats, mice and guinea pigs were of similar susceptibility to the inhibitory action of DAS on aerobic phosphorylation These differences between the *in vitro* and *in vivo* effects of DAS suggest that a difference in the rate of detoxification in the various species is responsible for the variation in susceptibility to the toxic agent rather than variations in the concentrations of sensitive enzymes

The strong inhibitory action of DAS on phosphate esterification *in vivo* and *in vitro* gives support to the possibility that some of the acute toxic effects of this compound may result from inhibition of this important biochemical reaction In support of such a possibility was the finding that the tissues which showed biochemical and pathological changes after DAS, namely the liver and kidney, are the same tissues in which DAS produced a depletion of phosphocreatine

SUMMARY

1 *In vitro* studies demonstrated that dimethylaminobenzenediazo sodium sulfonate (DAS) inhibits the succinic dehydrogenase activity of rat liver and kidney after a preliminary incubation of the enzyme with the rodenticide However, no inhibition of the succinic dehydrogenase activity of the tissues of rats was observed at 5 hours after the administration of 30 mgm /kgm of DAS No inhibitory effect by DAS was observed on cytochrome oxidase, malic dehydrogenase and fumarase

2 The oxidation of glucose by rat brain homogenates was inhibited *in vitro* by concentrations of DAS above $1 \times 10^{-6}\,M$, however, no depression of glucose oxidation by brain tissue from DAS poisoned rats was observed The respiration of liver tissue was inhibited *in vitro* and *in vivo* by DAS. The toxic agent had no effect on the rate of anaerobic glycolysis by rat liver slices and brain homogenates

3 DAS had no effect on the oxidation of fumarate, succinate, malate and oxalacetate by liver slices taken from rats 5 hours after the administration of 30 mgm /kgm of the toxic compound However, the stimulation of respiration of liver slices by pyruvate and citrate was depressed both *in vitro* and *in vivo* by DAS

4 The phosphocreatine of rat liver and kidney tissue was depleted within 5 hours after the administration of 30 mgm /kgm of DAS while the phosphocreatine of skeletal muscle and cardiac muscle was unaffected by this dose of the compound Other changes in the distribution of acid-soluble phosphorus compounds in the liver and kidney of DAS poisoned rats consisted of a slight decrease in glucose 1-phosphate and a small increase in glucose-6-phosphate, phosphopyruvate and triose phosphate

5 DAS exerted a marked inhibitory effect on the aerobic phosphorylation of creatine by rat, mouse and guinea pig kidney homogenates Aerobic phosphorylation was also inhibited in the tissues of animals given lethal doses of DAS The inhibition *in vivo* was dependent upon the dose of the compound administered and varied with the species susceptibility to DAS

REFERENCES

1. Stollé, R.: Berichte, 45: 2682, 1912.
2. Elliott, K. A. C., and Henry, M.: J. Biol. Chem., 163: 361, 1946.
3. Elliott, K. A. C., and Henry, M.: J. Biol. Chem., 163: 351, 1946.
4. Deutsch, W.: J. Physiol., 87: 560, 1936.
5. Schneider, W. C., and Potter, V. R.: J. Biol. Chem., 149: 217, 1943.
6. Potter, V. R.: J. Biol. Chem., 165: 311, 1946.
7. DuBois, K. P., and Potter, V. R.: J. Biol. Chem., 150: 185, 1943.
8. Umbreit, W. W., Burris, R. H., and Stauffer, J. F.: Manometric Techniques and Related Methods for the Study of Tissue Metabolism. 2nd printing, Burgess Publishing Co., Minneapolis, Minn., p. 160.
9. Potter, V. R.: J. Biol. Chem., 169: 17, 1947.
10. Potter, V. R., and DuBois, K. P.: J. Gen. Physiol., 26: 391, 1943.
11. Loomis, W. F., and Lipmann, F.: J. Biol. Chem., 173: 807, 1948.

John Auer
1875–1948

It is with profound sorrow that we record the death on April 30, 1948 of Dr. John Auer from a cardiac attack, at St. Mary's Hospital, St. Louis, Missouri, which he had entered about a week previously for a physical examination. Dr. Auer's passing removes from the Society for Pharmacology and Experimental Therapeutics one of its active organizers and distinguished members who exerted a strong influence in the upbuilding of the Society and in the promotion and application of the science of American pharmacology.

Dr. Auer was born in Rochester, New York, on March 30, 1875. His college preparation for his field was obtained at the Universities of Michigan and Johns Hopkins; he received his B.S. from the former in 1898 and his M.D. from the latter in 1902. After finishing his formal course in medicine, he served for a year as House Officer at the Johns Hopkins Hospital, following which he became a Fellow in the Rockefeller Institute for Medical Research, continuing there as Assistant, Associate, and Associate Member until 1921. During the years 1906–7 he was sent to the Harvard Medical School for special training in physiology, where he also served as Instructor in Physiology. In 1921 he accepted the Professorship of Pharmacology in the St. Louis University School of Medicine and was its Departmental Chairman until his death. From 1924 he also served continuously as Pharmacologist to the St. Mary's Hospital, of St. Louis. During the first World War he joined the Reserve Corps of the Army, in which service he attained the rank of Major.

Dr. Auer contributed much to both physiological and pharmacological sciences. Some of his studies at the Rockefeller Institute were done in conjunction with the late Samuel J. Meltzer. His scientific papers were of a varied nature and number about 150, among which were investigations on the heart, the kidney, the liver, the gall bladder, connective tissue, digestion, respiration, reflexes, functional disturbances caused by anaphylaxis, tetanus, war gases, and the physiological action of various drugs. During his war service he was the first to use magnesium sulfate intravenously as a relaxant in a case of tetanus.

In addition to being an organizer of the Society for Pharmacology and Experimental Therapeutics, he was its secretary from 1912 to 1917 and its president from 1924 to 1927. He was also a member of the Association of American Physicians, Society for Experimental Biology and Medicine (being its vice president from 1917–18), the American Physiological Society, American Association for the Advancement of Science, the Harvey Society, the St. Louis Academy of Sciences and the St. Louis Medical Society. A few weeks before his death he received honorary membership in the St. Louis Society of Anesthesiologists "for his pioneering work in the field of anesthesiology" and because his "continued interest has proved a source of stimulation to the younger members in this field and has contributed greatly to this branch of medical science."

Dr. Auer was the embodiment of the true investigator whose imaginative insight, perseverance and powers of analysis and synthesis were of a remarkably high order, his devotion to truth uncompromising. These fine qualities he brought to his students, together with his unselfishness and a vast knowledge in related fields, thus developing a breadth of view and an intelligence that is essential for the scientific physician. As a tribute to his outstanding qualities as a teacher and as an evidence of the esteem of the Lambda Chapter of the Phi Beta Pi Medical Fraternity it presented, in 1944, to the St. Louis University School of Medicine, the "John Auer Lectureship" which enabled the school to bring to it distinguished scientists from various fields.

It is difficult to describe briefly Dr. Auer "the man" because of his versatility. He was devoted to his family and friends, an enthusiastic gardener, an ardent reader of the classics, spending much time with the French, German, and Latin authors, a critic in literature, art, and music, and a painter whose Saturdays always included a sketching trip which ended with a visit to his old friends in frames on the walls of the Art Museum. In addition to his deep love for the truth, he possessed an intense sympathy for mankind, and an ardent desire to aid in the creation of a better world. His leadership and fellowship will be missed by men in all walks of life.

George B. Roth

A COMPARISON OF THE EFFECTIVENESS OF DIHYDROERGOTAMINE (DHE-45) AND DIHYDROERGOCORNINE (DHO-180) IN THE PREVENTION OF CARDIAC IRREGULARITIES DURING CYCLOPROPANE ANESTHESIA[1]

WILLARD D BENNETT, KARL GUSTAV DHUNER[2], AND O SIDNEY ORTH

Departments of Anesthesiology and Pharmacology, University of Wisconsin Medical School, Madison, Wisconsin

Received for publication November 3, 1948

In a pharmacological evaluation of dihydroergotamine methanesulfonate (DHE-45) reported by Orth and Ritchie (1), it was found that 0 4 mgm of the drug per kgm completely inhibited cardiac irregularities produced by the injection of a standard dose of epinephrine into dogs anesthetized with cyclopropane Similar protective activity with an equal dosage of dihydroergocornine (DHO-180) was reported by Orth, Capps and Suckle (2) The purpose of the present study is a comparison of the relative effectiveness of these two drugs in the prevention of cyclopropane epinephrine induced arrhythmias and also a preliminary note regarding trials and comparisons of DHO-180 and DHE-45 to prevent spontaneous cardiac irregularities arising clinically during cyclopropane anesthesia

METHODS Ten dogs and 4 monkeys (*Macacus rhesus*) were used in this comparative study The animals were anesthetized with cyclopropane and oxygen mixtures and maintained on approximately 30 per cent cyclopropane in oxygen by an endotracheal to and fro absorption method After equilibrating the animals on this mixture for 20 to 30 minutes, epinephrine was administered by a standard technic (3)

During the injection period electrocardiograms were recorded every 10 seconds In the second minute tracings were made every 15 seconds and then records were made at intervals for a period of 5 minutes from the beginning of the epinephrine injection The duration of ventricular tachycardia thus could be established for any given animal The possibility of an adrenolytic action of cyclopropane (4) also was determined for each animal

At subsequent periods with intervals of at least 3 days, the animals were anesthetized as above and given DHO or DHE, the drugs being administered separately to each animal on different days Doses of 0.2 mgm of the drugs per kgm were diluted to a total volume of 5 cc with normal saline and injected intravenously at the rate of 1 cc each 10 seconds Electrocardiograms were taken at appropriate intervals during these injections. After allowing 5 minutes to elapse following administration of the DH compound, the standard dosage of epinephrine previously found to be effective in eliciting ventricular tachycardia was given If 0 2 mgm /kgm of DH compound failed to protect against cardiac irregularities, some of the animals were given a subsequent 0 2 mgm /kgm within 10 minutes of the first dose for the purpose of more accurate comparison of the two drugs At 10 to 20 minute intervals after these injections, the standard dosage of epinephrine was repeated until cardiac irregularities again occurred or until 2 hours had elapsed after administration of the DH compound Thus the amount and duration of protection could be determined for comparison of the drugs

---

[1] Supported in part by the Research Committee of the Graduate School from funds supplied by the Wisconsin Alumni Research Foundation and in part by a grant from Sandoz Pharmaceuticals, New York

[2] Resident in Anesthesiology from Helsingborg, Sweden

In the *Macacus rhesus* when spontaneous irregularities occurred and normal rhythm could not be restored by inflation of the lungs with oxygen and decrease in depth of anesthesia, one of the DH compounds was injected intravenously. In all instances the irregularities occurring spontaneously were eliminated within 1 minute from the start of the injection. Thus it was possible to give epinephrine injections subsequently and determine the duration of protection.

In the preliminary clinical use of DHE and DHO for the protection of cardiac irregularities, 9 control and 23 test patients have been observed under a variety of operative conditions. The usual premedication of atropine or scopolamine alone or in combination with small doses of morphine sulfate was used. The three standard electrocardiographic tracings were taken, after which the DH compound was injected intravenously or intramuscularly. Initial dosage was 1 mgm. total amount. For succeeding patients this was increased to 5.5 mgm. of DHE or to 4 mgm. of DHO. Only 1 mgm. doses have been employed for intramuscular use. One to 3 minutes were used for the intravenous injections, during which time electrocardiographic records were taken. Two to 3 minutes following the injections, records were obtained again for the three standard leads. The induction of anesthesia with a cyclopropane-oxygen mixture was started within 5 minutes after completion of DH compound injection. Maintenance of anesthesia was with cyclopropane and oxygen using the to-and-fro absorption technique either with or without tracheal intubation.

The electrocardiographic beam, usually lead II, was observed continuously and tracings were made whenever cardiac irregularities were anticipated or seen. The usual graphic anesthesia record of pulse, blood pressure, respiratory rate and depth of anesthesia, along with other notations for correlation with the stage of the operation, was kept by the anesthetist.

RESULTS. In tables 1 and 2 are summarized the laboratory data. It can be noted from them that DHO unquestionably is a better protective agent in the 4 monkeys studied and that it appears to have some advantage over DHE in dogs. The adrenolytic effect of cyclopropane did not occur within the times noted in table 2 for duration of protection.

The clinical results up to the present time are summarized in table 3. Not only were arrhythmias more varied and frequent subsequent to the use of DHE than after the use of DHO, but those which arose were of greater possible danger. Three of the patients given DHE developed periods of ventricular tachycardia and in two of these instances it occurred within 10 minutes of the administration of the drug.

Oxygenation appeared to be good at the time tachycardia occurred and a relative overdose of cyclopropane seemed to be the cause. One of the 13 patients given DHO showed one short burst of ventricular tachycardia which occurred 30 minutes after the injection of 3 mgm. of the drug and at a time when there was cyanosis due to respiratory depression.

Nausea in 2 patients and vomiting in 1 occurred during the intravenous injection of DHO. Nausea or vomiting did not occur with the intravenous administration of up to 5.5 mgm. of DHE. The dosages were injected during a period of 3 minutes.

With the use of DHO there was a striking absence of the elevation of blood pressure that frequently occurs during the induction of cyclopropane anesthesia and there was a tendency for the systolic and diastolic pressure to become lower and remain lower than usual during the maintenance of anesthesia. This was not true in the patients treated with DHE.

Six of the 13 individuals given DHO exhibited periods of bradycardia with rates of under 50 per minute during cyclopropane anesthesia. None of the patients given DHE had such a degree of bradycardia.

Two of the individuals given DHO had a moderate bradycardia for 6 to 12 hours post-operatively. Otherwise there were no unusual post-operative effects.

Intestinal activity was of interest. Intraperitoneal operations were performed on 2 of the patients given DHO and observations were possible within 15 minutes of the start of the anesthetic. In both instances there was a marked contraction

TABLE 1

The protective action of Dihydroergotamine and Dihydroergocornine on cyclopropane-epinephrine tachycardia in dogs and monkeys

ANIMAL NO.	EPINEPHRINE DOSE	DURATION OF VENTRICULAR TACHYCARDIA		
		Control epinephrine response	DHE-45 (0 2 mgm / kgm) and epinephrine	DHO 180 (0 2 mgm / kgm) and epinephrine
		Dogs		
	mgm /kgm	*seconds*	*seconds*	*seconds*
1	.005	10	0	0
2	.01	15	0	0
3	.01	50	0	0
4	.01	20	0	0
5	.01	70	0	0
6	.02	10	Burst	0
7	.01	10	0	0
8	.01	50	40	Burst
9	.01	20	20	0
10	.01	70	0	Bursts
		Monkeys		
1	.001	20	Bursts*	0*
2	.001	20	0*	0*
3	.005	40	120	Bursts
4	.005	50	40	0

* *A dosage of 0.01 mgm./kgm. of epinephrine was used in these tests.*

of the gastro-intestinal tract. A cholecystectomy was done on one of the DHE treated patients. Only the gallbladder was adequately observed and it was found to be small and contracted.

Discussion. The number of dogs studied in this series is not sufficient and the differences between the protective ability of the two drugs in a given animal are too small to be certain that one drug is better than the other. Admittedly there is some day-to-day variation in the response of the individual animal. On the other hand, because of the clear-cut difference in the amount and duration of protection between DHO and DHE in the 4 monkeys, it seems fair to state that DHO is the better drug for this species.

In the dog and monkey, DHO, as well as DHE, prevents cyclopropane-epinephrine induced cardiac irregularities without harmful side effects. These

TABLE 2

Effects of dihydroergocornine and dihydroergotamine on cardiac irregularities produced deliberately in the dog and in the monkey

ANIMAL NO.	DH-COMPOUND		PROTECTION TO CYCLOPROPANE-EPINEPHRINE CARDIAC IRREGULARITIES*		REMARKS
	Drug injected	Dosage	Dosage of epinpehrine	Duration of protection	
Monkeys					
		mgm./kgm.	*mgm./kgm.*	*min.*	
1	DHE	0.2	0.01	None	Better protection with DHO
	DHO	0.2	0.01	70	
2	DHE	0.2	0.01	51	Better protection with DHO
	DHO	0.2	0.015	94	
3	DHE	(0.2 + 0.2)	0.005	None	Better protection with DHO
	DHO	(0.2 + 0.2)	0.005	38	
4	DHE	0.2	0.005	None	Better protection with DHO
	DHO	0.2	0.005	49	
Dogs					
1	DHE	0.2	0.005	58	Questionable advantage to DHO
	DHO	0.2	0.005	75	
2	DHE	0.2	0.01	111	Questionable advantage to DHO
	DHO	0.2	0.01	120+	
3	DHE	(0.2 + 0.2)	0.01	None	No advantage to either drug
	DHO	(0.2 + 0.2)	0.01	None	
4	DHE	0.2	0.01	None	No advantage to either drug
	DHO	0.2	0.01	None	
5	DHE	0.2	0.01	122	Approx. same protection with both drugs
	DHO	0.2	0.01	124	
6	DHE	(0.2 + 0.2)	0.02	28	Better protection with DHO
	DHO	0.2	0.02	68+	
7	DHE	(0.2 + 0.2)	0.01	23	Better protection with DHO
	DHO	0.2	0.01	54	
8	DHE	(0.2 + 0.2)	0.01	None	No advantage to either drug
	DHO	(0.2 + 0.2)	0.01	None	
9	DHE	(0.2 + 0.2)	0.01	None	No advantage to either drug
	DHO	(0.2 + 0.2)	0.01	None	
10	DHE	0.2	0.01	60	Better protection with DHE
	DHO	0.2	0.01	None	

* Ventricular tachycardia, ventricular premature contractions, or bundle branch block.

drugs also will eliminate spontaneous irregularities which occur in monkeys during cyclopropane anesthesia.

The clinical results up to the present time are far from being conclusive. Optimum dosage, route of administration, duration of action, and side-effects of these drugs for use during cyclopropane anesthesia in man can only be determined after many more patients are studied.

If the DH compounds do prove to be of value in the treatment or prevention of spontaneous cardiac irregularities arising during cyclopropane anesthesia, the results in these 23 clinical cases indicate that DHO would be the better drug to use. Kurtz, Bennett and Shapiro (5) made electrocardiographic observations on 41 patients during anesthesia with cyclopropane. Four of these patients developed ventricular tachycardia of relatively long duration. In the one instance that ventricular tachycardia occurred during the use of DHO in 13 patients, its duration was only a matter of a few seconds. Thus, the definite impression has been gained that DHO affords some protection in man.

TABLE 3

The effect in patients of the use of DHO-180 and DHE-45 to prevent spontaneous cardiac irregularities as observed and recorded electrocardiographically during cyclopropane anesthesia

NUMBER OF PATIENTS	TOTAL DOSAGE INJECTED	CARDIAC IRREGULARITIES OCCURRED	NO CARDIAC IRREGULARITIES OCCURRED
	DHO-180		
	mgm.		
3	1.0	1	2
7	2.0	4	3
3	4.0	1	2
	DHE-45		
2	1.0	1	1
3	2.0	2	1
1	3.0	1	0
1	5.0	1	0
3	5.5	2	1

Certain aspects of the sympathicolytic activity of DHE-45 and DHO-180 have been studied by Bluntschli and Goetz (6). They conclude that DHO is the first known ergot derivative to act in a purely sympathicolytic fashion in man and they suggest that its site of action is in the higher sympathetic centers, i.e., the medulla and/or the hypothalamus. In the study of the mechanism of cyclopropane sensitization (7), it was found that tachycardia does not appear after decerebration, the production of lesions in the pons, or bilateral sympathectomy. These findings suggest that DHO might be a suitable drug to control spontaneous cardiac irregularities occurring in man during cyclopropane anesthesia.

Up to the present time it appears unlikely that a standard dosage of DHO or DHE can be determined for clinical usage which will prevent cardiac irregularities without producing disturbing side effects. A more reasonable aim would be

to determine a dose that would prevent the serious arrhythmias that occasionally occur in a well managed anesthetic. Whether the effects on blood pressure, pulse rate, and intestinal motility prove to be a hazard or an aid to clinical anesthesia is purely speculative at this time.

CONCLUSIONS

1. Dihydroergocornine (DHO-180) appears to be a more effective drug than dihydroergotamine (DHE-45) for the prevention of cyclopropane-epinephrine induced cardiac irregularities in dogs and monkeys.
2. The compounds stop spontaneous cardiac arrhythmias occurring during cyclopropane anesthesia in the *Macacus rhesus*.
3. Preliminary clinical trials in 23 patients are described.

Acknowledgment for the help of Dr. Henry M. Suckle and Mr. Robert T. Capps in some of the experiments is gratefully extended.

REFERENCES

1. ORTH, O. S., AND RITCHIE, G.: THIS JOURNAL, 90: 166, 1947.
2. ORTH, O. S., CAPPS, R. T., AND SUCKLE, H. M.: Fed. Proc., 6: 361, 1947.
3. ALLEN, C. R., STUTZMAN, J. W., SLOCUM, H. C., AND ORTH, O. S.: Anesthesiol., 2: 503, 1941.
4. STUTZMAN, J. W., AND ALLEN, C. R.: Proc. Soc. Exper. Biol. and Med., 47: 218, 1941.
5. KURTZ, C. M., BENNETT, J. H., AND SHAPIRO, H. H.: J. A. M. A., 106: 434, 1936.
6. BLUNTSCHLI, H. J., AND GOETZ, R. H.: Am. Heart J., 35: 873, 1948.
7. ALLEN, C. R., STUTZMAN, J. W., AND MEEK, W. J.: Anesthesiol., 1: 158, 1940.

THE EFFECT OF TOPICALLY APPLIED ANTIHISTAMINIC DRUGS ON THE MAMMALIAN CAPILLARY BED[1]

THOMAS J HALEY AND D HARRIETTE HARRIS

Medical School, University of California at Los Angeles

Received for publication November 3, 1948

INTRODUCTION In a recent review of the pharmacology of the antihistaminic drugs, Loew (1) stated, "It will be important to determine the extent to which the action of antihistamine drugs is referable to a direct effect upon capillaries " This statement leads to the present investigation in which it was decided that topical application of these drugs without pre- or post histamine medication might show the site of action of the antihistaminic compounds insofar as the capillary bed itself was concerned

EXPERIMENTAL The meso appendix of Wistar strain rats was exteriorized and arranged for perfusion and microscopic observation according to the method of Chambers and Zweifach (2) The animals used weighed from 135–220 gm (average 150 ± 10 gm) The anesthetic, sodium pentobarbital 45 mgm /kgm intraperitoneally, was given in a total volume of not more than 0 15 cc to reduce any untoward effects such as those described by Zweifach *et al* (3) Further, as required to maintain the same depth of anesthesia additional anesthetic was given, usually not more than 0 05 cc of the pentobarbital solution The solutions of the drugs in Locke Ringer's were prepared prior to use and maintained at 37 5° ± 0 2°C until applied to the preparation The perfusion fluid, Locke Ringer's containing 1 per cent gelatin, was allowed to drip on the membrane at a temperature of 37 5° ± 0 2°C maintained by an electrically heated and thermostatically controlled water bath of 12 l capacity All animals were standardized as to vasoconstrictor response of the capillary bed by the topical application of 0 1 cc of 1 2 $\times 10^{6}$ solution of epinephrine and direct observation of the precapillary sphincters of a selected vessel Although all the vascular elements in the field were observed, all subsequent evaluations were made upon this vessel in a given animal and those which were not responsive were discarded The antihistaminics and control drugs were made up in molar solutions so that better comparison of their vasoconstrictor potency could be obtained The periods of observation after applying the drugs and washing to remove the drug were 5 and 10 minutes, respectively Epinephrine, 0 1 cc 1 2 $\times 10^{6}$, was applied before and after each dilution of the drug to check not only the normality of the membrane but also to determine accurately the type of response observed Further, to be certain that the drugs were acting at the same site as histamine, this drug was topically applied to the membrane at a dose of 0 1 cc of a 0 0001 molar solution, this being the dose necessary to increase the rate of flow in the vascular bed without causing any deleterious effects in the animals In order to rule out the effect of acetylcholine, this drug was also tested, and it was necessary not only to wash the membrane but to apply Pavatrine 0 1 cc of 0 01 molar solution to obtain again a response to epinephrine Further, to be certain that the effect of the drug was not due to the solvent used, 0 1 cc of water was applied to the membrane after the study of the responses of each animal was completed Also, when the response observed was doubtful, it was checked by applying either diphenhydramine or pyranisamine 0 1 cc of a 0 01 molar solution A control group was also tested with benzazoline because this drug, which contains the imidazole group, was known to be a potent vasodilator

---

[1] This paper is based on work performed under Contract No AT-04 1 GEN 12 with the Atomic Energy Commission for the University of California at Los Angeles

RESULTS. The effects observed with the various drugs tested are given in tables I to VI in which the drugs are classified as to chemical structure. Further, lateral histograms (see tables) are used to enable the potency to be rapidly estimated. In the dilutions which were effective, the degree of local vasoconstrictor activity of the drugs was equivalent to that produced by the similar local effects

TABLE I

Ethanolamine derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
Benadryl Diphenhydramine	β-dimethylamino ethyl benzhydryl ether HCl	255.35	*5/5	5/5	5/5	4/5	1/5	Precapillary sphincters completely closed at 0.001 *M*
Linadryl A-446	β - morpholino - ethyl benzhydryl ether HCl	297.33	5/5	5/5	0/5	0/5	0/5	Precapillary sphincters closed at 0.1 *M*
Decapryn Doxylamine	Benzyl - pyridyl-methyl - ethyoxy-dimethyl - amine succinate	270.36	5/5	5/5	1/5	0/5	0/5	Precapillary sphincters closed at 0.1 *M*
SC 1694	β-dimethylamino ethyl benzhydryl ether salt of 8-chloro-theophyllin	255.35	0/5	0/5	0/5	0/5	0/5	Vasodilator (See table VI)
Prep. 204	Benzhydryl-oxymethyl imidazoline HCl	266.33	5/5	5/5	5/5	3/5	0/5	Precapillary sphincters closed at 0.001 *M*
Tastromin 929F Thymoxyethyldiethylamine	2 - isopropyl - 5 methyl phenoxyethyl diethylamine HCl	249.38	5/5	5/5	5/5	3/5	0/5	Precapillary sphincters closed at 0.001 *M*. Capillary hemorrhage in one animal

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

of epinephrine. However, diphenhydramine, pyranisamine, phenindamine and phenergan caused a definite decrease in the blood flow throughout the entire field. This was observed as a blanching of the membrane, and indicates that these drugs at a dilution of 0.0001 *M* are more potent vasoconstrictors than epinephrine at 1:2 × 10^6. In general, the vasoconstrictor activity of these drugs agrees with both the experimental and clinical results thus far reported.

DISCUSSION The vasodilatation produced by such drugs as SC 1898 and SC 1742 is readily explained by their similarity to acetylcholine which also had a like

TABLE II

Thenyl derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0 1 M	0 01 M	0 001 M	0 0001 M	
Diatrin	N dimethyl N' - phenyl N' - (2 thienyl) methyl ethylenediamine HCl	260 39	*5/5	5/5	1/5	0/5	0/5	Precapillary sphincters completely closed at 0 1 *M*
Tagathen Chlorothen citrate	N dimethyl N' - pyridyl N' 5 chlorothenyl ethylenediamine citrate	293 81	5/5	5/5	2/5	0/5	0/5	Precapillary sphincters closed at 0 01 *M* Drug caused capillary hemorrhage in 3 animals at 0 1 *M*
Chlorothen	N dimethyl N' pyridyl N 5 chlorothenyl ethylenediamine HCl	293 81	5/5	4/5	3/5	0/5	0/5	Precapillary sphincters closed at 0 01 *M* Drug caused capillary hemorrhage in 3 animals at 0 1 *M*
Bromothen	N dimethyl N' pyridyl N' 5 bromothenyl ethylenediamine HCl	337 27	4/5	4/5	1/5	0/5	0/5	Precapillary sphincters completely closed at 0 1 *M*
Thenylene Histadyl Methapyrilene	N dimethyl N' - pyridyl N' thenylethylenediamine HCl	259 36	5/5	5/5	2/5	0/5	0/5	Precapillary sphincters closed at 0 01 *M* Drug caused capillary hemorrhage in one animal at 0 1 *M*

* The ratios signify the number of active vasoconstrictor responses over the number of animals used

action upon the capillary bed Furthermore, these three compounds as well as histamine decreased the stickiness of the white cells causing them to enter the streamline flow Epinephrine and the other antihistaminics caused the white cells to adhere to the vessel walls

The vasodilatation produced by SC 1694 was probably due to the 8-chlorotheophyllin being more potent than the diphenhydramine part of the molecule. In particular this drug has a more potent action upon the larger vessels in the

TABLE III

Ethylenediamine derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION 1 M	0.1 M	0.01 M	0.001 M	0.0001 M	REMARKS
Neoantergan† Pyranisamine	N'-p-methoxy-benzyl-N'-pyridyl N-dimethyl ethylene diamine maleate	285.40	5/5*	5/5	5/5	5/5	0/5	Precapillary sphincters completely closed at 0.001 *M*
Pyribenzamine Tripelennamine	N'-benzyl-N'-pyridyl-N-dimethyl-ethylenediame HCl	255.37	5/5	4/5	3/5	1/5	0/5	Precapillary sphincters completely closed at 0.01 *M*
Neohetramine Thonzylamine	N-p-methoxy-benzyl-N'-pyramidyl-N-dimethyl ethylene-diamine HCl	287.40	5/5	5/5	5/5	5/5	3/5	G.I. infection sensitizing the preparation to the drug
" "	" "		5/5	5/5	0/5	0/5	0/5	Precapillary sphincters closed completely at 0.1 *M*
1571 F	N'-phenyl-N'-ethyl-N-diethylethylene diamine HCl	220.35	5/5	5/5	5/5	5/5	0/5	Precapillary sphincters closed at 0.001 *M* but not as potent as Neoantergan
Antistine Phenazoline	N'-phenyl-N'-benzylamino-methyl-imidazoline HCl	265.35	5/5	5/5	5/5	3/5	0/5	Precapillary sphincters closed at 0.001 *M* but not as potent as Neoantergan

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

† Lessens effect of epinephrine, histamine, and H_2O.

preparation. In regard to vasodilatation by direct action on the muscular coating of the blood vessels, theophyllin is the most potent of all the xanthine drugs.

The finding that acetylcholine in high dilution produced a permanent vasodilatation unless neutralized by Pavatrine was to be expected because of the predominant role of the cholinergic fibers in the nervous control of the intestinal tract.

The inactivity of benzazoline, which has been shown to be a potent vasodilator on the capillaries of the rabbit's ear (4) and hind limb preparations (5), may have been due to the animal used (the rat). However, Littner (6) reported that benzazoline had no peripheral vasodilator effect in the toad, dog, rabbit and rat. Further, Braun (7), after using the rat, concluded that the site of action of the drug was proximal to, but not on, the capillaries themselves. Regardless of the exact site of action it is known that benzazoline definitely inhibits both injected epinephrine (8, 9) and sympathin released by adrenergic nerve stimulation (10–12). This is important because we observed no epinephrine inhibition or reversal after topical application of benzazoline to the rat meso-appendix capillary bed. Histamine, which acts directly upon smooth muscle, caused dilation and phenazoline and 204 both caused vasoconstriction. All four of these compounds contain the imidazole group and the latter two are analogues of Antergan and diphenhydramine, respectively. Thus, from a structural viewpoint it appears that the aromatic substituents at the opposite end of the aliphatic chain play a more important part than the imidazole group insofar as vasoconstrictor potency is con-

TABLE IV

Thiodiphenylamine derivatives

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
SC 1627 3015 RP	N-dimethylamino ethyl thiodiphenylamine HCl	270.26	5/5*	5/5	4/5	0/5	0/5	Precapillary sphincters closed at 0.01 *M*
Phenergan 3277 RP	N-dimethylamino - isopropyl thiodiphenyl - amine HCl	284.41	5/5	5/5	5/5	5/5	0/5	Precapillary sphincters completely closed at 0.001 *M*. Equal to Neoantergan
SC 1923	N-methyl ethanol amino ethyl thiodiphenyl - amine HCl	300.25	5/5	5/5	5/5	0/5	0/5	Precapillary sphincters closed at 0.01 *M*
SC 1898	*N-dimethyl ethanol amino ethyl thiodiphenylamine bromide*	*395.20*	*0/5*	*0/5*	*0/5*	*0/5*	*0/5*	*Vasodilator (See* table VI)
SC 1742	N - trimethyl - amino ethyl thiodiphenyl - amine chloride	320.75	0/5	0/5	0/5	0/5	0/5	Vasodilator (See table VI)

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

cerned. Furthermore, the results herein presented indicate that the vasoconstrictor activity of the antihistaminic drugs is directly on the muscle cells of the precapillary sphincters and is not mediated through the adrenergic nerves. This is in accord with the statement of Wells and Morris (13) that an antihistaminic drug competes with histamine for its site of action.

TABLE V

Miscellaneous compounds

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
SC 887	Diethylamino-ethanol 9–10, dihydroanthracene carboxylate HCl	323.28	5/5*	5/5	5/5	0/5	0/5	Precapillary sphincters closed at 0.01 *M*
Trimeton Prophenpyridamine	1-phenyl-1-(2-pyridyl)-3-dimethylamino propane	240.34	5/5	5/5	4/5	0/5	0/5	Precapillary sphincters closed at 0.01 *M*
Pavatrine	Diethylamino-ethyl-7-fluorene carboxylate HCl	310.26	5/5	5/5	2/5	0/5	0/5	Precapillary sphincters closed at 0.1 *M*
Thephorin† Phenindamine	2-methyl-9-phenyl 2,3,4,9-tetra-hydro-pyridinolene hydrogen tartrate	260.34	4/5†	5/5	5/5	5/5	0/5	Animal died.† Precapillary sphincters closed completely at 0.001 *M*, equal to Neoantergan
Trasentine Adiphenine	Diethylamino-ethyl ester of diphenylacetic acid HCl	311.23	0/5	0/5	0/5	0/5	0/5	No effect on capillary bed
Distilled water			145/145					Complete blanching of the membrane

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

† Lessens effect of epinephrine and histamine.

From the results herein presented, it appears that drugs having a molecular weight from 249 to 287 are more potent than those of higher molecular weight. Further, it appears that substitution of a thenyl or halogenated thenyl group for the benzyl or pyridyl group decreases the vasoconstrictor potency, although the molecular weight may be maintained within the optimal range. Also, it can be seen that cyclization of the terminal amine group to produce a morpholine group

decreases the activity while cyclization to form the imidazole group has little effect on the potency as measured on the capillary bed.

In the rabbit and dog, increased capillary permeability has been measured by following the rate of extravasation of dyes or India ink after the intradermal injection of histamine. Diphenhydramine (14–17), tripelennamine (14, 18, 19) thonzylamine (16, 20, 21), pyranisamine (22–24), doxylamine (25) and Diatrin

TABLE VI

Vasodilator compounds

COMMON NAME	CHEMICAL NAME	MOLECULAR WEIGHT BASE	MOLAR CONCENTRATION					REMARKS
			1 M	0.1 M	0.01 M	0.001 M	0.0001 M	
Acetylcholine chloride	—	181.59	5/5*	5/5	5/5	5/5	5/5	Complete relaxation of capillary bed, no recovery on washing
SC 1742	N - trimethyl - amino ethyl thiodiphenyl amine chloride	320.75	5/5	5/5	5/5	5/5	5/5	Same but recovered on washing. Also see table IV. 3 animals did not respond to histamine or distilled water
SC 1694	β-dimethylaminoethyl benzhydryl ether salt of 8-chlorotheophyllin	255.35	5/5	5/5	5/5	0/5	0/5	No effect on capillaries. Relaxation of metarterioles and arterioles. See table I
Priscol Benzazoline	2-benzyl imidazoline HCl	160.21	0/5	0/5	0/5	0/5	0/5	No effect on capillary bed
Histamine	β - imidoazolyl -4 ethylamine HCl	111.09	0	0	0	0	$\frac{145}{145}$	Relaxation of vessels and increased blood flow through entire area

* The ratios signify the number of active vasoconstrictor responses over the number of animals used.

(26) are all able to diminish or prevent the local accumulation of dye which occurs after the intradermal injection of histamine. The other antihistaminics would be expected to have a similar action differing only in the doses required to prevent the extravasation of the dye. The wheal and flare reaction that occurs after the intradermal injection of histamine in man is also diminished or prevented by premedication with the antihistaminic drugs (27). The reduction of the tuberculin skin reaction after antihistaminic medication has also been reported (28, 29), as

has the reduction in the egg white edema in the rat (30, 31). Further, Halpern (32) found that capillary resistance was elevated in allergic, but not in normal, subjects after medication with phenergan, Antergan or pyranisamine. Phenergan was the most potent in this respect. The action of histamine in these cases is purely a local one and the doses of antihistaminics employed are within the range herein reported as causing vasoconstriction on the capillary bed. Further, it is logical to assume that whether histamine itself acts to relax the precapillary sphincters or has a general relaxant effect upon the other vessels of the peripheral vascular system (metarterioles and arterioles), the overall effect would be one of congestion ending in the loss of fluid into the interstitial spaces. Any mechanism which prevents this relaxation and congestion would tend to decrease capillary permeability. The vasoconstrictor action of the antihistaminic drugs on the precapillary sphincters reduces the flow through the capillary bed and thus would reduce capillary permeability and the extravasation of dyes, India ink or plasma into the surrounding tissues. Further, the observation that when histamine was applied to the capillary bed the cell wall and/or the leucocytes tended to be less sticky (enter the streamline flow of the erythrocytes) might lead one to assume that the leucocytes play a mechanical part in either decreasing or increasing capillary permeability. Both the antihistaminics and epinephrine caused an opposite effect, namely, that of increasing the stickiness of the cell wall and/or the white cells causing those cells to remain more firmly attached to the cell walls of the vessels. Furthermore, by acting as vasoconstrictors at the precapillary sphincters, the antihistaminics would prevent histamine from acting without decreasing the total amount of circulating histamine. Pellerat and Murat (33) have shown that after antihistaminic therapy the circulating histamine was increased 6 to 12 fold for more than 24 hours. Staub (34) showed that intravenous epinephrine increased blood histamine levels and prior medication with intravenous phenazoline diminished this rise. Thus blocking the action did not result in too great a decrease in the histamine blood level. Furthermore, after the antihistaminic effect has worn off, there is a tendency for histamine to again produce its vascular effects.

This discussion of the capillary action of histamine does not take into consideration the other vascular effects of the drug. However, it has been shown that vasodepressor action (20, 21, 25, 32, 35–51) and the vasoconstrictor action on the larger arteries (52) are all blocked by the antihistaminic drugs. Such action would support the belief that both histamine and the antihistaminics act at the same site and that there is a different muscular area concerned with each action.

SUMMARY

1. It has been shown that the antihistaminic drugs, not related to acetylcholine, have a vasoconstrictor action on the precapillary sphincters of the mammalian capillary bed, while histamine has a vasodilator action. This demonstrates that both drugs compete for the same site of action.

2. It has been shown that acetylcholine and antihistaminic compounds having the choline group exert a vasodilator effect upon the capillary bed. Further, al-

though washing the bed counteracts the effect of the latter compounds, it is neces sary to apply an antispasmodic drug to counteract the effect of the former

3 It has been shown that antispasmodic drugs have at best only a slight vasoconstrictor action upon the capillary bed

4 The vasodilator action of 8 chlorotheophyllin is more potent than the vasoconstrictor action of diphenhydramine However, the effect is not upon the precapillary sphincters but upon the vessels having more muscular coats (arterioles)

5 The vasoconstrictor effect produced by the antihistaminics on the capillary bed is produced at dilutions which are of the same order of magnitude as those obtained in animals or humans after oral or intravenous administration Further, such vasoconstriction explains the decrease in Trypan Blue extravasation in animals, the decrease in the wheal and flare reaction in humans and the reduction in the tuberculin skin reaction

6 The cyclization of the terminal amine group to form a morpholine group decreases the vasoconstrictor action of diphenhydramine However, cyclization to form the imidazole group does not decrease the vasoconstrictor action of an anti histaminic The substitution of a thenyl or halogenated thenyl group for either the phenyl or pyridyl group results in a decrease in vasoconstrictor action

ACKNOWLEDGMENT The authors wish to thank Parke, Davis and Co for the Benadryl and Linadryl, Ciba Pharmaceutical Products, Inc , for the Pyribenzamine, Antistine, 204, Priscol and Trasentin, Abbott Laboratories for the 929F, 1571F and Thenylene, Lederle Laboratories for the Chlorothen, Bromothen and Tagathen, Pyridium Corp for the Neohetramine, Merck and Co for the Neoantergan, Wm R Warner and Co for the Diatrin, Wm S Merrell and Co for the Decapryn, Dr J J Reuse for the Phenergan, Schering Corp for the Trimeton, Hoffman La Roche for the Thephorin, and G D Searle and Co for the Pava trine Sc 887 Sc 1742 Sc 1694, Sc 1627, Sc 1923 and Sc 1898 used in this study

REFERENCES

1 LOEW, E R Physiol Rev , 27 542, 1947
2 CHAMBERS, R , AND ZWEIFACH, B W Am J Anat , 75 173, 1944
3 ZWEIFACH, B W , HERSHEY, S G , ROVENSTEIN, E A , AND CHAMBERS, R Proc Soc Exper Biol & Med , 56 73, 1944
4 MEIER, R , AND MUELLER, R Schweiz Med Wschr , 69 1271, 1939
5 MEIER, R , AND MEYER R T Schweiz Med Wschr , 71 1206, 1941
6 LITTNER, M Sem Med (Buenos Aires), 60 794, 1944
7 BRAUN, H Derm Wschr , 115 1008, 1942
8 AHLQUIST, R P , AND WOODBURY, R A Fed Proc , 5 161, 1946
9 HERMAN, H , AND VIAL, H Compt rend soc de biol , 136 803, 1942
10 HERMAN H , JOURDON, F , AND BONNET, V Compt rend soc de biol , 135 1653, 1941
11 CHESS, D , AND YONKMAN, F F Fed Proc , 4 114, 1945
12 CHESS, D , AND YONKMAN, F F Proc Soc Exper Biol & Med , 61 127, 1946
13 WELLS, J A , AND MORRIS, H C Fed Proc , 4 140, 1945
14 DREISBACH, R H Fed Proc , 6 323, 1947, J Allergy, 18 397, 1947
15 LAST, M R , AND LOEW, E R THIS JOURNAL, 89 81, 1947
16 BUKANTZ, S C , AND DAMMIN, G J Science, 107 224, 1948
17 TRAUB, F B , FRIEDMAN, U , AND LANDSTADT, D J Allergy, 18 273, 1947
18 RENNICK B , CHESS, D , HAYS, H W , MATHIESON, D , MAYER, R L , AND YONKMAN, F F Fed Proc , 4 133 1945

19. Mayer, R. L., and Kull, F. C.: Proc. Soc. Exper. Biol. & Med., 66: 392, 1947.
20. Scudi, J. V., Reinhard, J. F., and Dreyer, N. B.: J. Allergy, 19: 184, 1948.
21. Reinhard, J. F., and Scudi, J. V.: Proc. Soc. Exper. Biol. & Med., 66: 512, 1947.
22. Cullumbine, H.: Nature, 159: 841, 1947.
23. Bonvallet, M., and Decourt, P.: Compt. rend. soc. de biol., 138: 224, 1944; ibid, 138: 305, 1944.
24. Hunter, R. B.: Lancet, 252: 672, 1947.
25. Brown, B. B., and Werner, H. W.: J. Lab. Clin. Med., 33: 325, 1948.
26. Ercoli, N., Schlachter, R. J., Hueper, W. C., and Lewis, M. N.: This Journal, 93: 210, 1948.
27. Haley, T. J.: J. Amer. Pharm. Assoc., 37: 383, 1948.
28. Breton, A.: Compt. rend. soc. de biol., 137: 254, 1943.
29. Sarber, R. W.: Am. Rev. Tuberculosis, 57: 504, 1948.
30. Brown, B. B., and Werner, H. W.: Ann. Allergy, 6: 122, 1948.
31. Leger, J., and Masson, G.: Am. J. Med. Sci., 214: 305, 1947.
32. Halpern, B. N.: Acta Allergologica, 1: 3, 1948.
33. Pellerat, J., and Murat, M.: Compt. rend. soc. de biol., 140: 297, 1946.
34. Staub, H.: Helv. Physiol. Pharmacol. Acta, 4: C54, 1946.
35. Page, I. H., and Green, A. A.: Fed. Proc., 5: 78, 1946.
36. Bovet, D., Horclois, R., and Fournel, J.: Compt. rend. soc. de biol., 138: 165, 1944.
37. Halpern, B. N.: Arch. internat. Pharmacodyn. et de Therap., 68: 339, 1942.
38. Marsh, D. F., and Davis, A. J., Jr.: This Journal, 89: 234, 1947.
39. Dreyer, N. B., and Harwood, D.: Proc. Soc. Exper. Biol. & Med., 66: 515, 1947.
40. Sherrod, T. R., Loew, E. R., and Schloemer, H. F.: This Journal, 89: 247, 1946.
41. Lehmann, G., Hagen, E., Barbarow, G., and Roe, M.: Fed. Proc., 6: 350, 1947.
42. Dews, P. B., and Graham, J. D. T.: Brit. J. Pharmacol., 1: 278, 1946.
43. Yonkman, F. F., Oppenheimer, E., Rennick, B., and Pellet, E.: This Journal, 89: 31, 1947.
44. Wells, J. A., Morris, H. C., Bull, H. B., and Dragstedt, C. A.: This Journal, 85: 122, 1945.
45. Boquet, A.: Presse Med., 51: 93, 1943.
46. Ramanamanjary, W.: Compt. rend. soc. de biol., 138: 480, 1944.
47. Halpern, B. N.: Bull. soc. chim. biol., 29: 309, 1947.
48. Wells, J. A., Morris, H. C., and Dragstedt, C. A.: Proc. Soc. Exper. Biol. & Med., 61: 104, 1946.
49. Yonkman, F. F., Hays, H. W., and Rennick, B.: Fed. Proc., 4: 144, 1945.
50. Climenko, D. R., Homburger, E., and Messer, F. H.: J. Lab. Clin. Med., 27: 289, 1941.
51. Minard, D., and Rosenthal, S. R.: Proc. Soc. Exper. Biol. & Med., 44: 237, 1940.
52. Capraro, V.: Farm. sci. e tec. (Pavia), 2: 98, 1947.

STUDIES OF THE ACTIONS OF 4-AMINO-PTEROYLGLUTAMIC ACID IN RATS AND MICE[1]

FREDERICK S. PHILIPS AND JOHN B. THIERSCH[2]

WITH THE ASSISTANCE OF JOSEPHINE HAWRYLKO AND DORIS ROSENTHAL

Pharmacology Section, Division of Experimental Chemotherapy, Sloan-Kettering Institute for Cancer Research, and Department of Pharmacology, Cornell University Medical College, New York, N. Y.

Received for publication November 5, 1948

The cardinal signs of pteroylglutamic acid (PGA, folic acid) deficiency in mammals are disorders of hematopoiesis and of the digestive tract. Manifestations of severe deficiency include macrocytic anemia and granulocytopenia with hypoplasia of bone marrow involving erythro- and myelopoiésis. Megaloblastosis is found in monkeys and man. The lesions of the digestive tract include necrosis of the buccal and intestinal mucosa. Diarrhea is a feature. Current knowledge of the role of the vitamin in mammalian nutrition is exhaustively treated in a recent review by Jukes and Stokstad (1).

Attempts to develop structural analogs which antagonize the actions of PGA have been successful. Impure preparations of relatively low potency, presumably containing x-methyl analogues of PGA, were the first antagonists to accelerate and enhance the vitamin-deficiency when fed with PGA-deficient diets (2, 3, 4). As expected of competitive antagonists (5), the effects of these preparations were readily reversed by PGA. More recently a congener in which the 4-hydroxy group of PGA was replaced by an amino substitution (6) (4-amino-pteroylglutamic acid) was found to exert a fulminating toxic effect in laboratory animals. The lesions produced by this compound were difficult to prevent by simultaneous administration of PGA. Reports have appeared concerning its toxicity in several laboratory species (7, 8, 9) and further interest in the compound followed the announcement of its inhibitory actions against Rous sarcoma in young chicks (10) and acute leukemia in children (11). It appeared, therefore, of importance to explore the sites of its action and to assess whether the lesions produced by the agent could be considered to result from an antagonism of folic acid. For these reasons the study here reported was initiated in mice and rats. A subsequent report will deal with its actions in dogs (12).

PROCEDURE. Albino mice (AKM strain) and rats (Wistar strain) of both sexes served as experimental subjects. The animals had been raised and were maintained during the investigations on standard laboratory diets. With exceptions, mentioned below, the weights of mice were between 17.5 and 22.5 grams and of rats, between 100 and 200 grams.

---

[1] This investigation was supported by research grants to the Sloan-Kettering Institute from the U. S. Public Health Service, Division of Research Grants and Fellowships, and from the American Cancer Society.

[2] Neale Research Pathologist, University of Adelaide, S. Australia; National Cancer Institute Fellow, U. S. Public Health Service.

Solutions of 4-amino-PGA[3] were prepared fresh daily for immediate use in 0.9 per cent NaCl. They were made from ampules containing measured samples which had been neutralized before drying, or by the addition of 2 molar equivalents of $NaHCO_3$ to weighed amounts of the free acid. Doses were administered in the constant volume of 0.5 cc. per 20 gram mouse and 1.0 cc. per 100 gram rat. Standard procedures, as recommended by Wintrobe (13), were used to investigate the blood of rats. Samples of 2 or more cc. of blood were obtained in heparinized syringes at time of sacrifice from deep cuts across the axillary vessels of stunned rats. Bone marrow samples were obtained from long bones. Femoral shafts were connected to small syringes by means of rubber tubing and their contents expelled by air-pressure. Marrow smears were stained by the Jenner-Giemsa technic. When possible blocks of marrow were fixed in Vandergrift's reagent and, after embedding, stained with hematoxylin-phloxin-eosin and Giemsa. Representative samples of spleen, liver, duodenum, jejunum, ileum, colon, mesenteric lymph node, sternum, and thymus were treated likewise.

RESULTS. *Toxicity.* The LD_{50} of 4-amino-PGA in animals receiving single doses, as estimated graphically (14) from the data of table 1, was 1.9 ± 0.3 mgm. per kgm. in mice and 4.5 ± 1.4 mgm. per kgm. in rats. Several interesting features of the toxic actions may be noted in table 1. Although the agent was administered parenterally in doses which exceeded the LD_{50} at least tenfold, no animal succumbed earlier than the third day. Oral administration in rats was as effective as parenteral injection. Furthermore, 4-amino-PGA, when given daily in fractions of the lethal dose, appeared to be almost as toxic in mice and possibly more toxic in rats than when given in single doses.

Antagonism of 4-amino-PGA. Attempts to reduce toxicity by treatment of animals with PGA met with limited success. Since the daily dosage of PGA employed was near the maximum tolerated by mice, it was not possible to explore the antagonism of the agents beyond the limits shown in table 2. Within this range the LD_{50} of 4-amino-PGA was raised several fold by repeated administration of the vitamin. A similar result was obtained with large doses of pteroyltriglutamic acid (PTGA). It is interesting to note that the course of fatal intoxication was not appreciably altered in those animals which succumbed in spite of treatment with either PGA or PTGA. Most succumbed during the third or fourth day following 4-amino-PGA administration.

In view of possible interrelationships among the actions of PGA, the anti-pernicious-anemia factor in liver, and thymine (1) a few observations were made to assess the effects of the two last mentioned substances on the toxicity of 4-amino-PGA. When used as described for PGA and PTGA in table 2, refined liver extract and thymine in daily doses of 10 units per kgm. and 500 mgm. per kgm., respectively, failed to alter the effects of both 12.5 and 3.1 mgm. per kgm. of the toxic agent.

Course of Intoxication. During the first 24 hours following the administration of fatal doses of 4-amino-PGA the behavior and appearance of mice and rats were unaltered. Moderate losses in weight were noted by the end of the first day. Subsequently debilitation set in and progressed steadily to the extent that in-

[3] The authors are grateful to the late Dr. Y. SubbaRow and Dr. T. H. Jukes and their associates in the Lederle Laboratories for their generous provision of both ampuled preparations and bulk samples of 4-amino-PGA, PGA, pteroyltriglutamic acid, and purified liver extract.

toxicated animals lost approximately 20 per cent of their initial weight within 72 hours. Severe, watery diarrhea appeared 48 hours after poisoning. The feces

TABLE 1

Toxicity of 4-amino-PGA in mice and rats

SPECIES	ROUTE OF ADMINISTRATION	NO. OF INJECTIONS	DOSAGE		MORTALITY	DAY OF DEATH		
			Single	Total		3 to 4	5 to 7	8 to 14
			mgm./kgm./day	mgm./kgm.				
Mouse	Intraperitoneal	1	100	100	6/6	6	—	—
		1	25	25	6/6	6	—	—
		1	12.5	12.5	34/34	30	3	1
		1	6.3	6.3	11/12	10	1	—
		1	3.1	3.1	26/40	22	4	—
		1	1.6	1.6	9/18	8	1	—
		1	0.8	0.8	2/18	1	1	—
		1	0.4	0.4	3/6	—	3	—
		1	0.2	0.2	3/6	1	1	1
		5	1.6	8.0	6/6	2	4	—
		7	0.8	5.6	12/12	2	9	1
		7	0.4	2.8	7/12	—	5	2
		7	0.2	1.4	2/6	—	1	1
		7	0.1	0.7	0/3	—	—	—
		7	0.05	0.35	0/3	—	—	—
Rat	Intraperitoneal	1	40	40	6/6	3	3	—
		1	20	20	5/6	2	3	—
		1	10	10	5/6	2	3	—
		1	5	5	4/6	1	2	1
		1	2.5	2.5	2/6	—	1	1
		1	1.25	1.25	0/6	—	—	—
		4	1.0	4.0	4/4	3	1	—
		4	0.5	2.0	4/4	2	2	—
		8	0.25	2.0	6/10*	—	6	—
		8	0.125	1.0	0/6†	—	—	—
	Oral	1	40	40	5/6	3	2	—
		1	20	20	5/6	2	2	1
		1	10	10	4/6	3	1	—
		1	5	5	2/6	1	—	1
		1	2.5	2.5	2/6	2	—	—
		1	1.25	1.25	0/6	—	—	—

* Two surviving animals sacrificed on seventh day for hematologic and pathologic study; one of pair exhibited severe debilitation and diarrhea.

† All sacrificed on tenth day for hematologic and pathologic study; at this time the weights in percent of initial were as follows: 89, 101, 104, 104, 110, 133.

were yellowish-brown in color and, terminally, grossly stained with blood. The diarrhea persisted as a prominent feature of fatal intoxication in both mice and rats and contributed to their dehydrated and depressed appearance at death.

Animals surviving minimum lethal doses exhibited transient retardation in growth or actual loss of about 5 per cent of body weight during the first week. (Untreated animals of similar age from the same colony gain consistently 1 to 2 per cent of body weight per day.) Their subsequent recovery was rapid.

Pathology. The lesions caused by single, fatal doses of 4-amino-PGA were studied in rats receiving 40 mgm. per kgm. intraperitoneally. The animals were sacrificed at various intervals as noted in table 3 ("acute" group).

Effects of chronic administration of the agent were observed in a limited group of animals receiving either 0.25 or 0.125 mgm. per kgm. per day (PI and PII, table 3) and in a larger series of 15 males and 14 females which received the doses shown in figure 1 and table 3 (groups I to V). Animals of the latter series were sacrificed when they failed to gain or lost weight for at least 4 consecutive days.

TABLE 2

Effect of single doses of 4-amino-PGA in mice treated with PGA and PTGA

TREATED WITH	DOSE OF 4-AMINO-PGA	MORTALITY
	mgm./kgm.	
PGA*	12.5	15/18
	3.1	2/18
	1.6	0/6
PTGA†	12.5	4/6
	6.3	1/6
	3.1	3/12
	1.6	0/6

* 47 mgm./kgm. administered intraperitoneally once daily at −2, −1, 0, 1, and 2 days.
† 500 mgm./kgm., once daily, as for PGA.

Only 4 animals of this series, all females, succumbed before sacrifice. The remaining females exhibited signs of poisoning before the end of the first 7 weeks and were included among the first 4 groups of animals (groups I to IV, table 3). Group V consisted of 8 male rats. Their gain in body weight was not seriously impaired until the initial dose had been increased fourfold.

1) *Lesions of blood and hematopoietic tissue.* The femoral marrow of rats receiving 40 mgm. per kgm. was converted from a greyish-red, gelatinous staff of material to a darker, more fluid substance by the twelfth hour after treatment. Progressive liquefaction of the marrow occurred until at 72 hours only purple fluid could be expelled from the femur. In the peripheral blood marked granulocytopenia and reticulocytopenia and a moderate lymphopenia developed simultaneously. In table 3 are shown changes in peripheral blood and bone marrow. It is evident that within 48 hours erythropoiesis and myelopoiesis were severely inhibited. The elevated values for lymphoid cells in the femoral contents taken from 48- and 72-hour animals were due to replacement of marrow tissues by blood. Hemoconcentration became evident at 48 hours when diarrhea first appeared.

Ill-defined areas of degeneration were observed in sternal and femoral marrow as early as 6 and 12 hours after the administration of 40 mgm. per kgm. of 4-amino-PGA. After 24 and 48 hours the sinus and capillary network of the marrow became prominent due to depletion in erythro- and myelopoiesis. Between 48 and 72 hours the hematopoietic tissues largely vanished from the marrow leaving a network of capillaries and sinusoids filled with blood. Capillary endothelial lining and histiocytes were prominent in smears and sections. The remainder of the original marrow consisted for the most part of eosinophils, mega-

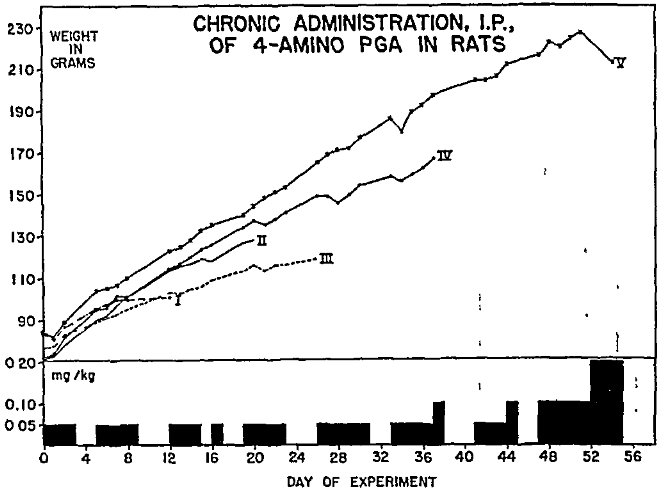


FIG 1. GROWTH IN BODY-WEIGHT OF RATS RECEIVING REPEATED, INTRAPERITONEAL INJECTIONS OF 4-AMINO PGA. DAILY DOSAGE INDICATED BY SOLID COLUMNS ALONG ABSCISSA

karyocytes, and a few basophil normoblasts and erythroblasts. Pyknosis was common in these cells. In the marrow smears lymphocytes were prominent due to admixture of peripheral blood since no lymph follicles or aggregations of these elements were found in sections.

In animals receiving 0.25 or 0.125 mgm. per kgm. of 4-amino-PGA daily for periods of 5 to 9 days (groups PI and PII, table 3) the hematological changes resembled those described above in the "acute" group 48 and 72 hours after poisoning However, changes in hematopoietic tissues developed less rapidly and were less severe when small daily doses of 0.05 mgm. per kgm. were given (groups I to V, figure 1 and table 3). No significant changes were found in animals of group I and only one of group II and one of group III evidenced marrow depletion. Moderate depletion was found in the 3 animals of group IV.

TABLE 3

Hematological data from rats treated with 4-amino-PGA

(Number of animals used indicated in brackets)

GROUP	DOSAGE	NUMBER OF INJECTIONS	DAY OF SACRIFICE		PERIPHERAL BLOOD W.B.C.	PMN.	MNC.	Retic.	Hcrit.	M.C.V.	M.C.H C.	FEMORAL MARROW Condition	PMN-N	PMN-EOS	M Myel.	Myelc.	Mibl.	Lymph	Normbl.
	mgm / kgm / day				*1 c mm. × 10⁻³*	%	%	%	*cc./100 cc.*	*μ³*	*g /100 cc.*		%	%	%	%	%	%	%
Control				Mean Range	6 9 (11) 2 7–13 9	18 (11) 12–29	82 (11) 71–88	11 (6) 7–17	38 4 (6) 37 4–41 4	56 (6) 52–62	34 (6) 31–38	S (11)	33 (12) 10–57	6 (12) 2–20	9 (12) 4–13	8 (12) 3–16	2 (12) 1–5	9 (12) 3–21	35 (12) 16–70
Acute	40	1	5		20 4	15	85	5	46 0	—	33	F	79	7	5	0	0	1	7
					5 5	52	48	5	31 8	—	31	F	22	4	49	0	4	2	17
					8 9	5	95	10	44 6	59	36	SF	67	4	5	2	1	4	17
					7 7	10	90	24	38 4	71	32	SF	28	2	8	4	2	11	45
			1		5 3	28	72	10	32 7			F	40	20	25	7	1	1	6
					2 1	16	84	10	38 5			F	34	12	16	9	1	20	8
					16 8	5	95	2	51 8	58	31	F	45	6	5	5	3	32	4
					8 7	6	94	8	40 0	56	31	F	28	4	10	5	5	12	36
			2		2 6	8	92	6	52 8		34	F	2	7	3	0	0	84	4
					8 5	50	50	2	52 5		34	F	34	8	8	5	1	40	4
					2.1	2	98	1	52 4	48	29	F	3	9	0	2	0	82	4
					1 4	3	97	2	39 2	56	35	F	2	14	0	0	1	81	0
			3	Mean Range	1 6 (6) 1 0– 2 5	1 (8) 0–4	99 (8) 96–100	0 (2)	59 3 (2) 56 9–61 6	58 (1)	34 (2) 33–34	F (6)	3 (8) 0–13	6 (8) 3–10	2 (8) 0–5	2 (8) 0–7	0 (8) 0–1	82 (8) 67–92	2 (8) 0–3
P I	0 25	5	7		6 3 2 2	14 6	86 94					S F	55 6	4 15	3 2	22 2	4 0	7 67	3 6
P II	0 125	8	10	Mean Range	3 4 (6) 1.3– 5 2	9 (6) 1–35	91 (6) 65–99					F (6)	13 (6) 2–32	13 (6) 5–22	11 (6) 6–23	12 (6) 4–18	4 (6) 1–8	39 (6) 29–61	8 (6) 3–15
I	0 05	7–12	12–20	Mean Range	8 5 (4) 6 5–11 8	51 (5) 25–67	42 (5) 25–75	15 (3) 8–22	38 3 (3) 30 3–50 0		31 (3) 23–39	S (4)	17 (4) 9–23	5 (4) 0–18	24 (4) 18–30	12 (4) 6–19	0 5 (4) 0–1	0 5 (4) 0–2	41 (4) 22–65
II	0 05	14	22	Mean Range	5 4 (4) 2 2– 9 9	24 (4) 6–56	76 (4) 44–94	8 (4) 2–15	35 9 (4) 32–43 5		34 (4) 31–38	S(3), F(1)	25 5(4) 16–43	7 (4) 2–13	14 (4) 8–17	11 5 (4) 7–16	7 5 (4) 5–10	5 (4) 1–16	29 (4) 18–34
III	0 05	15–17	26–28	Mean Range	11 3 (5) 4 4–18 3	23 (5) 6–40	75 (5) 60–94	10 (5) 4–17	43 0 (5) 35 0–50 5	60 (5) 52–71	31 (5) 23–34	S(4), SF(1)	37 (5) 31–42	4 5 (5) 2–8	14 (5) 8–22	7 (5) 3–11	0 (5)	2 (5) 1–3	35 (5) 27–41
IV	0 05–0 10	30	48	Mean Range	5 9 (3) 3 5– 8 2	16 (3) 6–30	84 (3) 70–94	7 (3) 4–10	40 0 (3) 37 8–44 2	51 (3) 52–57	33 (3) 32–34	S (3)	41 (3) 21–58	19 (3) 14–31	11 (3) 9–13	3 (3) 2–4	3 (3) 1–5	4 (3) 2–7	18 (3) 5–28
V	0 05–0 20	36 and 37	54 and 55	Mean .. Range .	3 2 (8) 0 8–10 7	10 (8) 0–37	90 (8) 73–100	0 (8) 0	46 5 (8) 37 0–59 0	59 (8) 52–64	32 27–35	SF(3), F(5)	16 (8) 0–53	30 (8) 8–38	7 (8) 2–17	4 (8) 2–9	1 (8) 0–2	38 (8) 17–53	4 (8) 1–7

Abbreviations

Hcrit, hematocrit
F, fluid
Lymph, lymphocytes
M.C.H C., mean corpuscular hemoglobin concentration
M C.V., mean corpuscular volume
Mibl., myeloblasts
M Myel., metamyelocytes
MNC., mononuclear cells, i e, lympho- and monocytes
Myelc, myelocytes
Normbl., normoblasts
PMN., polymorphonuclear granulocytes
PMN-EOS, polymorphonuclear eosinophils
PMN-N., polymorphonuclear neutrophils
S, solid
SF, semi-fluid
Retic., reticulocytes
W B C., white blood cell count

However, animals of group V within 9 days after elevation of daily dosage from 0.05 to 0.1 and 0.2 mgm. per kgm. showed advanced changes in blood and marrow consistent with the lesions found in the "acute" animals and in groups PI and PII.

2) *Lesions of the intestinal tract.* All animals had a natural oral mucosa and esophagus. However, the stomach and intestinal canal were filled in most cases with a yellow-brownish fluid and were often distended. Parts of the colon and rectum were spastically contracted. The fluid found in the gastro-intestinal tract was a transparent, viscid material, almost gelatinous in early stages but later more watery and plasma-like. Only in later stages were leucocytes and blood present.

Microscopic examination of tissues from small and large intestines, taken from animals as early as 6 and 12 hours after injection of 40 mgm. per kgm. of 4-amino-PGA, revealed venous hyperemia and marked dilatation of capillaries and venules in mucosa and submucosa. As a result the distal ends of villi were distended. Plasma extravasation was evident in the submucosa between the dilated vessels and the epithelium. The epithelium was enlarged partly by cytoplasmatic vacuolation. In some instances it had desquamated permitting plasma and lymph to flow into the lumen. After 24 and 48 hours these phenomena were more pronounced. Both surface and crypt epithelium showed, in addition to marked enlargement and vacuolation, extensive desquamation associated with rapid, often abnormal, regeneration of cells containing atypical, giant nuclei. Infiltration of villi and submucosa with neutrophils, lymphocytes and eosinophils began at 24 hours and, thereafter, increased progressively. Many polymorphonuclears mixed with desquamated epithelium could be found forming plugs in the crypts and were still present at a time when the marrow was almost completely free of granulocytes. After 72 hours both the small and large intestine showed extensive broadening and apparent shortening of villi or plicae due to extensive leucocytic infiltration, hyperemia, and edema. There were also small areas of fresh hemorrhages due to loss of the superficial parts of plicae.

In animals poisoned by chronic administration the extent of damage to the intestinal tissues paralleled the findings in bone marrow. Thus, groups PI and PII exhibited intestinal changes like those seen in animals of the "acute" group. Intestinal lesions were absent in Group I and only one of each of group II and III exhibited characteristic, early changes. Marked intestinal edema, desquamation of mucosa, and infiltration of leucocytes were noted in group IV. Finally, intestinal tracts of animals of group V were indistinguishable from those of experimental animals 72 hours after receiving 40 mgm. per kgm.

3) *The lymphoid system.* By the second and third days after the dose of 40 mgm./kgm. lymph nodes of the axilla, groin, and mesentery as well as lymph follicles of the spleen and lymph placques of the intestine decreased moderately in size. At the same time a decreased number of lymphocytes was found in the circulation. Such changes might be related to migration of lymphocytes into the wall of the gut where they were found in abundance. It is pertinent to note that, with the exception of cortical pyknosis of the thymus in rats 48 and 72 hours after 40 mgm./kgm., necrotic changes in lymphoid tissues were not observed.

The relatively moderate effect on lymphoid tissues is one of the outstanding differences between the actions of 4-amino-PGA and those of nitrogen mustard (15).

4) *Other lesions.* Apart from the lesions described above, a general venous congestion of all internal organs was noted.

5) *Complications.* In five per cent of rats receiving 4-amino-PGA by chronic administration, salmonella infections altered the typical course of poisoning. After gut lesions were established, an ascending infection took place with enlargment of the mesenteric lymph nodes, abscess formation, multiple fibrinoid necrosis in liver, spleen, kidney and lung, bronchitis and bronchopneumonia.

DISCUSSION. The derangements produced in rats by 4-amino-PGA include failure to gain weight, hypoplasia of bone marrow, and edema of the intestinal tract associated with desquamation and diarrhea. Each of these changes is found in folic acid deficiency and their combined appearance forms the classical syndrome (1). Nitrogen mustards and x-rays also produce lesions in bone marrow and intestinal tract but at the same time damage severely all lymphoid tissues. However, the fact that 4-amino-PGA has a more selective action in erythro- and myelopoietic tissues by comparison with its effects on lymphoid tissues is consistent again with the syndrome of folic acid deficiency (16). Moreover, intestinal changes following 4-amino-PGA and nitrogen mustard differ. Following fatal poisoning with nitrogen mustard the epithelium of the intestinal tract enlarges to a greater extent without desquamation and hyperemia and at the same time leucocytic infiltration of submucosa is not as marked as in 4-amino-PGA intoxication (17).

On the basis of the lesions produced by 4-amino-PGA, as described above, and its structural similarity to PGA it is reasonable to conclude that 4-amino-PGA acts as an antagonist of folic acid. This conclusion is supported by work on the growth of *Streptococcus fecalis R* (7, 9, 18). However, in mice (7), rats and chicks (9) 4-amino-PGA differs from an ideal metabolite-antagonist (5) in that its actions are not readily prevented or reversed by PGA. In this respect 4-amino-PGA is unlike x-methyl folic acid which acts as a competitive antagonist of PGA in rats, mice, and chicks (3, 4). In addition, the speed of onset and severity of lesions following administration of 4-amino-PGA exceeds by far the effect obtained with the same x-methyl antagonist. It is also to be noted that stomatitis found in rats receiving the reversible antagonist was missing in animals given 4-amino-PGA.

The discrepancies between the actions of 4-amino-PGA and those of an ideal metabolite-antagonist might be related to a marked affinity of the potent agent for the physiological loci of action of pteroylglutamic acid. On this basis the rapid onset and severity of lesions caused by the antagonist could be considered to result from an immediate and absolute folic acid deficiency in affected cells leading to their rapid degeneration and death. Accordingly the administration of folic acid might be relatively ineffective in preventing or reversing the toxic actions of 4-amino-PGA.

The failure to observe oral lesions in rats following administration of 4-amino-PGA corresponds to the findings in rats receiving folic acid deficient diets (16). However, such lesions are commonly found in patients treated with 4-amino-PGA

(11). Moreover, one might speculate that various antagonists of folic acid could differ sufficiently in distribution among the tissues of higher organisms such that at critical levels of dosage characteristic patterns of response could be expected.

SUMMARY AND CONCLUSIONS

1. The toxicity of 4-amino-pteroylglutamic acid (4-amino-PGA) has been studied in mice and rats following acute and chronic administration.
2. The agent was almost as toxic in mice and possibly more toxic in rats when given daily in fractions of the lethal dose.
3. The course of fatal intoxication was not altered by doses exceeding the LD_{50} at least tenfold.
4. The toxicity of 4-amino-PGA was reduced to only a limited extent in mice by simultaneous administration of large doses of either PGA or pteroyltriglutamic acid.
5. The syndrome produced in rats corresponded to a folic acid deficiency and consisted of loss of weight, hypoplasia of bone marrow, and intestinal lesions with diarrhea. The syndrome was rapid in onset and led quickly to death.
6. It is concluded that 4-amino-PGA produces an absolute immediate deficiency of pteroylglutamic acid (PGA). The affected cells degenerate rapidly, which makes reversibility by PGA improbable. Regeneration can be expected only from cells relatively insensitive to the actions of the agent.

REFERENCES

1. Jukes, T. H., and Stokstad, E. L. R.: Physiol. Rev., 28: 51, 1948.
2. Martin, G. J., Tolman, L., and Moss, J.: Science, 106: 168, 1947.
3. Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H.: Proc. Soc. Exper. Biol. and Med., 65: 368, 1947.
4. Franklin, A. L., Stokstad, E. L. R., Belt, M., and Jukes, T. H.: J. Biol. Chem., 169: 427, 1947.
5. Wooley, D. W.: Physiol. Rev., 27: 308, 1947.
6. Seeger, D. R., Smith, J. M., Jr., and Hultquist, M. E.: J. Am. Chem. Soc., 69: 2567, 1947.
7. Franklin, A. L., Stokstad, E. L. R., and Jukes, T. H.: Proc. Soc. Exper. Biol. and Med., 67: 398, 1948.
8. Minnich, V., and Moore, C. V.: Fed. Proc., 7: 276, 1948.
9. Oleson, J. J., Hutchings, B. L., and SubbaRow, Y.: J. Biol. Chem., 175: 359, 1948.
10. Woll, E.: Trans. N. Y. Acad. Sci., Ser. 2, 10: 83, 1948.
11. Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A.: New England J. Med., 238: 787, 1948.
12. Thiersch, J. B., and Philips, F. S.: To be published.
13. Wintrobe, M.: "Clinical Hematology", 2nd. Edition, Lea and Febiger, Philadelphia, 1946.
14. Miller, L. C., and Tainter, M. L.: Proc. Soc. Exper. Biol. and Med., 57: 261, 1944.
15. Kindred, J. E.: Arch. Path., 43: 253, 1947; Graef, I., Karnofsky, D., Jager, V. B., Krichesky, B., and Smith, H. W.: Amer. J. Path., 24: 1, 1948.
16. Endicott, K. M., Daft, F. S., and Ott, M.: Arch. Path., 40: 364, 1945; Spicer, S. S., Daft, F. S., Sebrell, W. H., and Ashburn, L. L.: Public Health Reports, 57: 1559, 1942.
17. Karnofsky, D. A., Thiersch, J. B., Patterson, P. A., and Ridgway, L. M.: Anat. Rec., 100: 50, 1948.
18. Smith, J. M., Jr., Cosulich, D. B., Hultquist, M. E., and Seeger, D. R.: Trans. N. Y. Acad. Sci., Ser. 2, 10: 82, 1948.

THE PROTECTIVE ACTION OF VARIOUS AGENTS AGAINST CHLOROFORM-EPINEPHRINE VENTRICULAR FIBRILLATION

R. A. HUGGINS, R. A. MORSE, C. A. HANDLEY, AND M. LA FORGE

Department of Pharmacology, Baylor University College of Medicine

Received for publication November 8, 1948

The production of fatal ventricular fibrillation in barbitalized dogs under chloroform anesthesia by the intravenous injection of large doses of epinephrine is a well established phenomenon (1).

It has been reported that certain agents presumed to produce coronary vasodilatation such as quinacrine (1) papaverine (2), or nitrites (3), the intravenous administration of procaine (4, 5, 6, 7), quinidine (8), or adrenolytic substances such as priscol (9, 10) and dibenamine (10, 11, 12) are useful in the prophylaxis of this phenomenon.

In the present series 3 groups of agents were tested for their protective influence against the chloroform-epinephrine effect on the dog's heart: a) coronary vasodilators: sodium nitrite (3 dogs), aminophylline (6 dogs), papaverine (3 dogs), and quinacrine (2 dogs).

b) those decreasing myocardial excitability: procaine (11 dogs), quinidine sulfate (6 dogs).

c) adrenolytic agents: priscol (4 dogs), dibenamine (2 dogs).

The production of ventricular fibrillation is not an invariable result of the administration of intravenous epinephrine during chloroform anesthesia to barbitalized dogs. For this reason, the absence of fibrillation alone cannot be used as an index of the protecting properties of the agent administered as a test drug.

In the present series, 21 dogs manifested ventricular fibrillation following the first or subsequent administrations of chloroform-epinephrine. Of these 21 dogs, 18 (85 per cent) demonstrated a tachycardia with increasing ventricular excitability immediately preceding the onset of fibrillation. For purposes of this study these changes are considered to represent "prefibrillation" changes. To protect properly against the onset of chloroform-epinephrine ventricular fibrillation, an agent should prevent the onset of "pre-fibrillation" changes as well as fibrillation.

METHOD. Medium sized dogs anesthetized with barbital 250 or 300 mgm. per kgm. were used. The common carotid artery was cannulated and the blood pressure recorded on moving photographic paper by means of a Hamilton optical manometer (13).

The drug to be tested was injected into an exposed femoral vein, and enough time allowed to elapse for the blood pressure and pulse rate to become stabilized following the injection. Chloroform was administered by means of a positive pressure anesthetic machine and face mask, the rate of flow regulated so as to minimize the fall in blood pressure as much as possible. Following 5 minutes of chloroform administration, epinephrine 0.02 mgm. per kgm. was rapidly injected into the femoral vein, and the chloroform immediately discontinued.

Tracings were obtained as follows: at the time the test drug was administered and continued until the maximum effect was manifest; at the onset of the chloroform inhalation

and at one minute intervals during chloroform administration, and at the time of epinephrine injection, continuing until ventricular fibrillation ensued or a definite trend back toward normalcy had been established Control tracings were made prior to the administration of the test drug in each instance

The chloroform epinephrine administration was repeated at regular intervals without further administration of the test drug in the majority of the dogs who survived the first administration

RESULTS Table I summarizes the maximum rhythm changes produced in each dog in the series and indicates the time intervals elapsing between the first and subsequent chloroform epinephrine administrations

1 Sodium nitrite, 10 mgm per kgm was given to 3 dogs (# 16, 17, 18) In all cases there was an associated fall in blood pressure, but no appreciable change in cardiac rate or rhythm Following the injection of epinephrine to dog # 16 there was no increase in blood pressure but rhythm disturbances appeared as demonstrated by complete heart block with idio ventricular rhythm followed by coupled ventricular premature contractions Normal rhythm was restored after 4 minutes The chloroform epinephrine administration was repeated twice with identical results Dogs # 17 and 18 responded to the epinephrine injection with an abrupt rise in blood pressure and pulse rate The tachycardia was followed by increased ventricular irritability in both cases, and dog # 17 went on to fatal ventricular fibrillation Dog # 18 returned to a normal mechanism after 30 seconds of ventricular premature contractions Repetitions of the chloroform epinephrine administration to dog # 18 up to 60 minutes produced similarly bizarre tracings Neither dog # 16 nor 18 went into ventricular fibrillation on any of the injections

2 Aminophylline, 10 mgm per kgm was given to 6 dogs producing a fall in blood pressure in 4 (# 9, 11, 12, and 13), and a rise in 2 (10 and 14) The cardiac rate and rhythm were unchanged Following the epinephrine injection there was an abrupt rise in blood pressure and heart rate in all six In 2 (# 10 and 13) the tachycardia proceeded to ventricular fibrillation In the remainder (# 9, 11, 12, 14) there were many ventricular premature contractions lasting from 2 to 6 minutes Repetition of chloroform epinephrine administration to dog # 12 produced ventricular fibrillation Repetitions to dogs # 9, 11, and 14 produced pre fibrillation changes similar to those following the first administration None of these 3 dogs developed ventricular fibrillation, even after an interval of $1\frac{1}{4}$ to 2 hours following the administration of the aminophylline

3 Papaverine, 5 mgm per kgm was given to 3 dogs (# 20, 21, 22) The administration was followed by an increase in pulse pressure due to a fall in diastolic pressure in all three Following the epinephrine injection there was no significant rise in blood pressure in any case Dog # 20 demonstrated a tachycardia followed by ventricular premature contractions and fibrillation, dog # 21 demonstrated a tachycardia followed immediately by ventricular fibrillation, and dog # 22 had coupled ventricular premature contractions followed by fibrillation

4 Quinacrine, 20 mgm per kgm was given to 2 dogs (# 27 and 28) In both cases there was an associated fall in blood pressure Following the ad-

TABLE I

Summary of maximum effect on rhythm following chloroform-epinephrine

DRUG	DOG	FIRST CHLOROFORM-EPINEPHRINE ADMINISTRATION			SUBSEQUENT CHLOROFORM-EPINEPHRINE ADMINISTRATIONS*		
		No irregularities (protection)	Pre-fibrillation changes. No fibrillation	Ventricular fibrillation	No irregularities (protection)	Pre-fibrillation changes. No fibrillation	Ventricular fibrillation
Chloroform - epinephrine alone with no protective substance	1		X			X	
	2			X			
	3			X			
Sodium nitrite 10 mgm./kgm.	16		X			XX	
	17			X			
	18		X			XXXX	
Aminophylline 10 mgm./kgm.	9		X			XX (1)	
	10			X			
	11		X			XX (2)	
	12		X				X (3)
	13			X			
	14		X			XXX (4)	
Papaverine 5 mgm./kgm.	20			X			
	21			X			
	22			X			
Atabrine 20 mgm./kgm.	27		X			X (5)	X (6)
	28		X				X (7)
Procaine 20 mgm./kgm.	4			X			
	6	X				XXXX (8)	
	7		X				X (9)
	15			X			
	29	X				X	X
	30		X			X	
	31			X			
	32			X			
	33			X			
	34			X			
	5			X			
Respiratory arrest following administration of procaine 25 mgm./kgm.							
Quinidine Sulfate 10 mgm./kgm.	25		X (3 sec. duration)		X		
	26	X				XX	
	35		X				X
	36	X					X
	37	X				X	
	38	X			XXXX (10)		

TABLE 1—*Concluded*

DRUG	DOG	FIRST CHLOROFORM EPINEPHRINE ADMINISTRATION			SUBSEQUENT CHLOROFORM EPINEPHRINE ADMINISTRATIONS*		
		No irregularities (protection)	Pre-fibrillation changes No fibrillation	Ventricular fibrillation	No irregularities (protection)	Pre fibrillation changes No fibrillation	Ventricular fibrillation
Priscol 10 mgm / kgm	24	X			X		
	39	X			XX		
	40	X			XX		
	42	X					
Dibenamine 20 mgm /kgm	23	X			X		
	41	X					

* At intervals of 15 minutes unless otherwise specified
(1) 20 and 35 minutes after initial administration
(2) 20, 90, and 110 minutes after initial administration
(3) 20 minutes after initial administration
(4) 20, 40, and 70 minutes after initial administration
(5) 10 minutes after initial administration
(6) 20 minutes after initial administration
(7) 10 minutes after initial administration
(8) 10, 30, 45, and 60 minutes after initial administration
(9) 20 minutes after initial administration
(10) 15 and 30 minutes, 1½, 2½, and 3½ hours after initial administration

ministration of chloroform epinephrine to dog #27 there was no hypertensive response, but a tachycardia was manifest followed by marked ventricular irregularity and A-V conduction changes Repetition of these agents produced an abrupt rise in blood pressure, tachycardia, and ventricular fibrillation Dog #28, following epinephrine, demonstrated a hypertensive response followed by ventricular premature contractions, ventricular tachycardia and tachysystole of 12 seconds duration Ten minutes later, readministration of chloroform-epinephrine produced fibrillation

5 Procaine, 20 mgm per kgm was given to 10 dogs with no associated change in rate, rhythm, or blood pressure Following the chloroform epinephrine administration there was an abrupt and significant rise in blood pressure in all cases Six of the dogs developed ventricular fibrillation Dogs #7 and 30 developed pre fibrillation changes, with return to normal mechanism in 3 and 2 minutes, respectively Dogs #6 and 29 responded to epinephrine with a sinus tachycardia demonstrating no irregularities in rhythm Repetition of the chloroform epinephrine administration to the dogs which had shown pre fibrillation changes (#7 and 30) reproduced identical changes in #30, and produced ventricular fibrillation in #7 Readministration of the chloroform epinephrine to dogs that had been protected (#6 and 29) produced marked pre-fibrillation changes, and a third administration to dog #29 resulted in ventricular fibrillation

Dog #5 was given 25 mgm procaine per kgm and immediately after the injection died

6. *Quinidine sulfate*, 10 mgm. per kgm. was given to 6 dogs, and was associated with a very slight fall in blood pressure and marked slowing of the rate in all. Following the epinephrine injection there was an abrupt rise in blood pressure of significant degree, and a rise in pulse rate to approximately the control level. There were no rhythm disturbances in 4 of the 6 dogs following the first chloroform-epinephrine administration. Dog #25 showed a very brief run of ventricular tachycardia lasting for 3 seconds with spontaneous reversion to the sinus mechanism. Dog #35 demonstrated definite pre-fibrillation changes following the first chloroform-epinephrine administration. The chloroform-epinephrine administration was repeated and produced ventricular fibrillation in 2 cases (#35 and 36), pre-fibrillation changes in 2 (#26 and 37), and no rhythm disturbances in 2 (#25 and 38). It should be noted that dog #25, which showed a 3-second run of ventricular tachycardia after the first chloroform-epinephrine administration was protected for the second. Dog #38 failed to show evidence of any rhythm disturbances up to 3½ hours after having received quinidine.

7. Priscol, 10 mgm. per kgm. was given to 4 dogs with a marked rise in systolic pressure and moderate increase in rate. Epinephrine was followed by a slight rise in systolic and a significant fall in diastolic pressure, and a tachycardia in all cases. There were no irregularities in rhythm. Chloroform-epinephrine was repeated at 15 minute intervals once (#24) or twice (#39, 40) with identical results.

Dibenamine, 20 mgm. per kgm. was given to 2 dogs with essentially no changes in blood pressure and slight slowing of the rate. After an interval of 30 minutes chloroform-epinephrine administration produced a tachycardia in both, but no rhythm disturbances were noted. Re-administration of chloroform-epinephrine after 15 minutes produced identical results.

Discussion. Of the 14 dogs receiving so-called "coronary vasodilators" 6 had ventricular fibrillation following the first chloroform-epinephrine administration, and the remaining 8 demonstrated pre-fibrillation changes of considerable severity. None were protected in the sense of preventing the changes which have been seen to immediately precede fibrillation. These findings are at variance with those of Melville (1, 3), who reported protection against chloroform-epinephrine ventricular fibrillation with coronary dilators.

The agents in the second group were variable. Procaine protected 2 dogs against arrhythmias following the first chloroform-epinephrine administration, 1 dog died following procaine administration alone, 2 showed marked pre-fibrillation changes and 6 manifested ventricular fibrillation. The protection of 2 out of 11 dogs, and the death due to procaine of 1 out of 11 dogs indicates that procaine is unreliable as a protective agent, findings in agreement with the conclusions of Wiggers and Wegria (4), and Nickerson (7), who found procaine to be of very little prophylactic value.

Quinidine sulfate in the dose employed had appreciable protective properties, a finding in agreement with Wegria and Nickerson (8). Four of the 6 dogs failed to show any of the arrhythmias after the first chloroform-epinephrine administration, which appeared following subsequent administrations in all but 1 (#38). One dog (#25) showed only a very transient pre-fibrillation change

which reverted to the sinus mechanism after only 3 seconds, changes which were insignificant for all practical purposes Dibenamine and priscol, adrenolytic agents, gave complete protection to 2 and 4 dogs, respectively, finding in agreement with previous reports (8, 9, 10, 12)

It has been reported pro (14) and con (1, 11) that an abrupt and definite rise in blood pressure is essential to the production of this type of ventricular fibrillation Twenty seven of the dogs showed a rise in systolic pressure in excess of 20 mm Hg, 8 of which showed no rhythm disturbances Eight dogs demonstrated no appreciable hypertensive response (less than 20 mm Hg), of which 5 had pre fibrillation changes or fibrillation, and 1 dog had a fall in systolic pressure (10 mm Hg) and fibrillated From this evidence it is apparent that the pressor effect of epinephrine is not related to the production of ventricular fibrillation during chloroform inhalation

SUMMARY

1 Three groups of agents were studied for their prophylactic effect against the production of ventricular fibrillation in barbitalized dogs given epinephrine during chloroform anesthesia

a So called coronary vasodilating agents sodium nitrite, aminophylline, papaverine, and quinacrine

b Agents decreasing myocardial excitability procaine and quinidine sulfate

c Adrenolytic agents dibenamine and priscol

2 Criteria for protection against ventricular fibrillation were defined so as to include prevention of increased ventricular excitability preceding fibrillation as well as prevention of fibrillation

3 Coronary dilating agents afford no protection

4 Procaine affords no practical protection

5 Quinidine sulfate affords appreciable and consistent protection

6 Adrenolytic agents afford complete protection

7 A pressor response to epinephrine is not necessary for the production of this type of ventricular fibrillation

REFERENCES

1 Melville, K I This Journal, 87 350, 1946
2 Melville, K I This Journal, 66 107, 1939
3 Melville, K I This Journal, 94 136, 1948
4 Wiggers, C J, and Wegria, R Am J Physiol, 131 296, 1940
5 Burstein, C T, and Marangoni, B A Proc Soc Exper Biol and Med, 43 210, 1940
6 Bittrich, N M, and Posers, W F Cur Res Anes and Anal, 27 181, 1948
7 Nickerson, M Fed Proc, 7 247, 1947
8 Wegria, R, and Nickerson, N D This Journal, 75 50, 1942
9 Ahlquist, R P Huggins, R A, and Woodbury, R A This Journal, 89 271, 1947
10 Nickerson, M, and Goodman, L S Fed Proc, 7 397, 1948
11 Garb, S, and Chenoweth, M B Fed Proc, 7 220, 1948
12 Garb, S, and Chenoweth, M B This Journal, 94 12, 1948
13 Hamilton, W F, Brewer, G, and Brotman, I Am J Physiol, 107 427, 1934
14 Shen, T C R, and Morri, R Arch Internat Pharmacodyn et de Therap, 64 58, 1940

EFFECTS OF MORPHINE, CODEINE, AND DILAUDID ON BLOOD FLOW

R. A. HUGGINS, C. A. HANDLEY, AND M. LA FORGE

Department of Pharmacology, Baylor University College of Medicine

Received for publication November 8, 1948

After the administration of morphine vomiting occurs regularly in the dog and nausea and vomiting occasionally in man. This action of morphine has been demonstrated to be central in origin and the result of a preliminary "stimulating" action on the vomiting center (1). Not so commonly recognized is the fact that after depression of the vomiting center has occurred, the assumption of an upright position may again precipitate nausea and vomiting. This has been observed to occur in the human with 0.3 to 0.5 mgm./kgm. of morphine (2). The fact that nausea and vomiting did not occur while in the horizontal position but that it could be repeatedly produced in the same individual on standing suggested the possibility that these phenomena were of circulatory origin.

METHODS. Dogs were anesthetized with sodium barbital 250 mgm./kgm. intraperitoneally. The blood pressure was obtained from the right carotid artery using an optical manometer (3) and blood flow to the leg or head was measured with a Gregg-Shipley rotameter (4). Chlorazol Fast Pink, 2 cc. of an 8 per cent solution per kgm. was used as the anticoagulant. For controls, simultaneous blood pressures and rate of flow were determined in the horizontal position and then the head or legs suddenly raised to an angle of approximately 45°. The drug was then given either intra-arterially (i.a.) into the distal arm of the flow meter or intravenously (i.v.) into the femoral vein and at varying intervals up to two hours blood flow and pressures were obtained in the horizontal position and at a 45° angle. In every dog but two, only one drug was used. The use of morphine after codeine, (LF 5), and after dilaudid, (LF 8A), were the two exceptions.

RESULTS. The elevation of either the head or hind legs of the dogs to a 45° angle during the control period had no detectable effect on either blood pressure or flow.

Morphine and codeine in the concentrations used when administered i.a. produced a marked increase in flow usually followed by a drop in pressure in both the head and leg regions. In measurements of head flow for example: codeine 0.1 mgm./kgm. increased the flow by 21 cc. and lowered the pressure from an average of 181/143 to 178/140 for about a minute and a half. This drop in pressure, while small occurred in all dogs receiving codeine. Morphine, 0.1 mgm./kgm., produced a greater increase in flow and fall in pressure than did codeine. The flow increased to a maximum of 27 cc. and the pressure fell to 143/98 from 190/148. The pressure with both morphine and codeine returned to the original level in 2–3 minutes. The flow returned toward normal in about the same period of time although in a few dogs the flow remained at a level of from 5–10 cc./min. above the control level for varying periods up to an hour. The same general response was observed with the leg although the absolute flow

of course was less The blood pressure and flow response to 0 01 mgm /kgm of dilaudid is more like codeine The drop in pressure is even smaller or may not occur and the increase in flow less, the increase averaging 10 cc and lasting about a minute to a minute and a half

Tables I and II show clearly that with morphine, but not with codeine or dilaudid, there is a significant drop in flow to either the head or leg region if

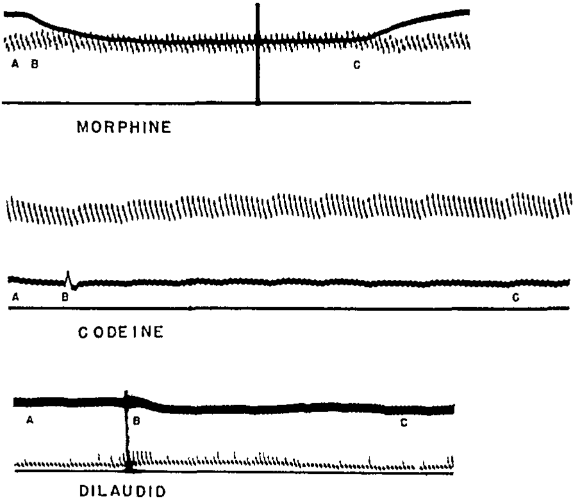


Fig 1 A, Control, Dog in Horizontal Position B, Head Elevated 45° C, Returned to Horizontal

that part is elevated 45° Dog HF 17 was the sole exception The decrease in flow occurs whether the morphine is given i a or i v Further, once this response becomes evident, increasing the amount of morphine administered does not increase the drop in flow With dog HF 11 a drop in flow of 13 cc /min occurred with 0 1 mgm /kgm and after 0 3 mgm /kgm the decrease in flow was still 13 cc /min Typical results are shown in fig 1

Lastly, dog LF 8A after receiving dilaudid with no apparent effect of blood flow on elevation was given 0 1 mgm /kgm of morphine, and on being raised 45° there was an immediate drop in flow of 19 cc /min The same thing was observed with dog LF 5 who had received 1 mgm /kgm of codeine and then 0 1 mgm /kgm of morphine The blood flow was reduced by 8 cc /min

TABLE I

Head flow—cc./min.

DOG	DRUG	DOSE	CONTROL	RAISED 45°	HORIZONTAL
		mgm./kgm.			
HF 8	Morphine	0.1	102	71	99
			99	68	98
HF 9	Morphine	0.3	46	30	50
			50	40	49
HF 10	Morphine	0.1	27	23	30
HF 11	Morphine	0.1	57	44	60
		0.1	64	34	67
		0.1	60	47	67
HF 17	Morphine	0.5	60	58	60
HF 18	Morphine	0.1	64	56	64
		0.1	72	56	73
			90	86	94
		0.3	62	54	68
			64	58	60
HF 12	Codeine	0.1	50	46	46
			38	41	38
		0.1	64	60	67
HF 12A	Codeine	1	32	30	36
HF 15	Dilaudid	0.01	78	79	79
		0.01	82	80	81
HF 16	Dilaudid	0.01	68	66	68
HF 16A	Dilaudid	0.01	69	70	70

TABLE II

Leg flow—cc./min.

DOG	DRUG	DOSE	CONTROL	RAISED 45°	HORIZONTAL
		mgm./kgm			
LF 3	Morphine	0.1	30	14	29
			34	23	28
LF 4	Morphine	0.1	31	22	30
			30	21	29
LF 4A	Codeine	1	35	34	36
LF 5	Codeine	1	24	22	25
	Morphine	0.1	26	18	24
LF 8	Dilaudid	0.01	59	56	61
			51	[illegible]	55
LF 8A	Dilaudid		62		60
			[illegible]		74
	Morphine				72

In the majority of dogs there was no effect on blood pressure when the flow decreased. A few showed a slight increase or decrease in blood pressure but never more than 3–4 mm. Hg.

Discussion:

That morphine has an antiemetic effect in dogs has been shown by Leake (5). In demonstrating this action, doses between 6 and 10 mgm./kgm. were used while the dosage range in these experiments was between 0.1 and 0.5 mgm./kgm. The antemetic action of morphine does not occur within the dosage range used here.

These data suggest that morphine interferes with the vascular compensatory mechanism that normally insures an adequate cerebral blood flow during postural changes. This inhibition only becomes evident on elevation of the dog and is shown by the inability of the circulatory system to compensate for the change in position and the result is a reduced flow to the elevated part. With the reduced minute volume occurring with morphine and the drop in flow which occurs if the head is elevated the anoxia resulting might be sufficient to "stimulate" the vomiting center.

It would be expected, if this is true, that dilaudid and codeine would be less likely to produce vomiting because the vasoconstrictor center is not inhibited as shown by the ability of the circulatory system to maintain flow to the elevated part.

SUMMARY

1. Elevation of the hind legs or head of a dog to 45° after the administration of morphine causes a significant reduction in blood flow to the part raised.
2. Morphine may produce a partial inhibition of the vasoconstrictor center.
3. Codeine and dilaudid lack this effect.

Acknowledgement. This investigation was added by a grant from the Bilhuber-Knoll corporation.

REFERENCES

1. Hatcher, R. A., and Weiss, S.: This Journal, 22: 139, 1923.
2. Handley, C. A.: Unpublished data.
3. Hamilton, M. F., Brewer, G., and Brotman, J.: Am. J. Physiol., 107: 427, 1934.
4. Gregg, D. E., Shipley, R. E., Eckstein, R. W., Rotta A., and Wearn, J. T.: Proc. Soc. Exper. Biol. and Med., 49: 267, 1942.
5. Leake, C. D.: This Journal, 20: 359, 1922–23

ANESTHETIC PROPERTIES OF SODIUM 5-ALLYL-5-(1-METHYLBUTYL)-2-THIOBARBITURATE (SURITAL) AND CERTAIN OTHER THIOBARBITURATES IN DOGS[1]

J. B. WYNGAARDEN,[2] L. A. WOODS, R. RIDLEY,[3] AND M. H. SEEVERS

Department of Pharmacology, University of Michigan, Ann Arbor

Received for publication November 10, 1948

Certain inherent disadvantages are common to all of those few barbiturates (Evipal, thiopental, Thioethamyl, Kemithal) which are detoxified with sufficient rapidity to make them useful for intravenous administration as anesthetic agents. Outstanding among these disadvantages are: (a) detoxication mechanisms become saturated if anesthesia is prolonged, so that these agents soon cease to retain their short acting characteristics and cumulative effects are noted; (b) certain reflexes, notably those having to do with the larynx, persist and are troublesome even in deep anesthesia; (c) respiration is very often seriously depressed; (d) these compounds, as is common to all barbiturates, lack the capacity to block specifically the central thalamic reception of painful stimuli; (e) it is generally believed that these compounds have an adverse effect upon cardiac activity.

The present study (1, 2, 3) is in search of an agent which might possess advantages over those compounds now in common use, particularly thiopental (Pentothal).

After a preliminary survey of several compounds which were made available to us by Dr. Bywater of the Parke, Davis and Company Research Laboratories, three were selected for further evaluation: sodium 5-allyl-5-(1-methylbutyl)-2-thiobarbiturate (Surital), sodium 5-ethyl-5-isoamyl-2-thiobarbiturate (Thioethamyl), and sodium 5-isopropyl-5-(2-methyl-2-pentenyl)-2-thiobarbiturate (B-10). See table I.

METHOD. With the exception of thiopental, which was used as the commercial sodium salt, these compounds were obtained as the acids and dissolved by neutralization with equivalent amounts of sodium hydroxide and buffered with fifty mgm. of sodium carbonate per gram of acid. Solutions of these various thiobarbiturates were used in concentrations between 0.75 and 5.0 per cent, calculated as acid weight.

In all experiments, except for the laryngeal spasm studies carried out on cats, the dog was used as the experimental animal. Administration in all instances was by vein and injection was made at a constant and uniform rate. Careful observations of the signs of anesthesia were recorded. The duration of anesthesia was arbitrarily fixed as the time from the onset of unconsciousness until the animal was able to stand erect when stimulated.

In the initial experiments all compounds were administered at a dose of 25 mgm./kgm. Sodium Thioethamyl was repeated at 50 mgm./kgm. because of its ineffectiveness at the lower dose level. Sodium B-10, being more potent, was used at a dose of 12.5 mgm./kgm.

---

[1] Supported by a grant from Parke, Davis and Company, Detroit, Michigan.

[2] Present address, Department of Medicine, Massachusetts General Hospital, Boston, Mass.

[3] Present address, Division of Anesthesiology, Mayo Clinic, Rochester, Minn.

After evaluation of the potency at the same dose level, namely 25 mgm /kgm., sodium thiopental and sodium Surital were administered to dogs in their respective ratios of potency, namely 1.0 to 1.5. The doses used were 10 mgm /kgm. for Surital and 15 mgm./kgm. for thiopental.

In order to evaluate the cumulative potentialities of these thiobarbiturates, small doses of each compound were injected at hourly intervals. Dosage of the several drugs was adjusted to produce approximately the same duration of anesthesia with the first injection.

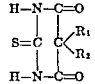
The effect of three thiobarbiturates, Surital, Thioethamyl, and B-10, upon the laryngeal reflex of the cat was determined according to the method of Burstein and Rovenstine (4). An attempt to produce laryngeal spasm in the dog by rectal dilatation was unsuccessful.

RESULTS. *Quality of Anesthesia.* Thiopental produced a smooth, rapid induction rarely accompanied by signs of stimulation or excitation. Satisfactory surgical anesthesia was produced with doses of 15 to 25 mgm./kgm. Emergence was fairly prompt and usually unaccompanied by excitation.

Surital effected an equally smooth induction, perhaps more rapid and with fewer signs of excitation. Doses of 10 to 20 mgm./kgm. resulted in good surgical anesthesia. Emergence was similar to that observed with thiopental but was somewhat more rapid.

TABLE I

Thiobarbituric acid derivatives

		R_1	R_2
	Thiopental Surital Thioethamyl B-10	$-CH_2-CH_3$ $-CH_2-CH=CH_2$ $-CH_2-CH_3$ $-CH(CH_3)-CH_3$	$-CH(CH_3)-CH_2-CH_2-CH_3$ $-CH(CH_3)-CH_2-CH_2-CH_3$ $-CH_2-CH_2-CH(CH_3)_2$ $-CH_2-C(CH_3)=CH-CH_2-CH_3$

With Thioethamyl induction was characterized by more marked and frequent stimulation as compared with thiopental and Surital. In addition Thioethamyl did not produce satisfactory muscular relaxation and emergence was slow with prolonged drowsiness and muscular weakness.

B-10 produced an undesirable amount of stimulation during induction although the type of anesthesia was quite adequate. Emergence was rapid, but the animals frequently showed much stimulation, irritability and excitation which often persisted for fifteen to thirty minutes.

Anesthetic Potency. The essential data concerning the duration of anesthesia with a given dose are shown in tables II and III. The approximate ratio of potency (thiopental = 1) of the four thiobarbiturates studied is thiopental, 1.0; Surital, 1.5; Thioethamyl, <0.5; and B-10, >1.5.

Cumulative Effect. Table IV compares the cumulative effects of the four thiobarbiturates. Injections were stopped when one or more dogs in any one series showed a duration of anesthesia exceeding sixty minutes. In each series every injection produced anesthesia of longer duration than the previous injection. Thioethamyl produced the most marked cumulative effect, followed by thiopental and lastly by Surital and B-10. Figure 1 depicts graphically the

increases of duration of anesthesia over those of the initial injection. A statistical analysis of the differences in third hour durations (following the fourth injection) is shown in table V.

Laryngeal Spasm. All four thiobarbiturates produced sneezing, hiccoughing, and coughing (supposedly characteristic of laryngeal spasm) in cats to much the same degree. There does not seem to be any apparent advantage in the use of one particular drug.

TABLE II

Durations of anesthesia

DRUG	CONCENTRATION OF SOL'N	DOSE (ACID WT.)	NO. OF DOGS	AV. WT. OF DOGS	MEAN DURATION OF ANESTHESIA	S.E.M	t_{VALUE}
	%	*mgm./kgm.*		*kgm.*			
Sod. thiopental............	5.0	25.0	22	8.8	74.4	7.1	10.47
Sod. Surital...............	2.5	25.0*	24	9.8	132.2	10.9	12.12
Sod. Thioethamyl.........	2.5	25.0	5	8.9	15.4	4.7	3.27
Sod. Thioethamyl.........	5.0	50.0	5	10.3	94.2	18.9	4.98
Sod. B-10..................	2.5	12.5	8	11.7	58.5	8.1	7.22

* Artificial respiration was necessary with a few animals to carry over an initial period of apnea.

TABLE III

Quality of anesthesia under equivalent doses of thiopental and Surital

DRUG	CONC. OF SOL'N	DOSE (ACID WT.)	NO. OF DOGS	AV. DURATION OF CORNEAL AREFLEXIA	AV. DURATION OF ANESTHESIA	COMMENTS
	%	*mgm./kgm.*		*min.*	*min.*	
Sod. thiopental...	3.0	15.0	8	8.5	26.0	Some restlessness on induction and emergence
Sod. Surital.......	2.0	10.0	8	7.3	26.6	No stimulation. Emergence and induction more rapid. The quality and depth of anesthesia equal

Discussion. The quality of anesthesia following intravenous administration of Surital in dogs is as satisfactory as that obtained with thiopental. As a result of undesirable reactions during induction and emergence Thioethamyl and B-10 are much less suitable for intravenous anesthesia than either thiopental or Surital. In addition, as will be discussed in subsequent paragraphs, Thioethamyl requires a larger dose because of its low level of potency and exhibits a marked cumulative effect.

A comparison of potency based on the duration of anesthesia assigning thiopental a value of 1.00 shows Surital with a potency of about 1.5 and Thioethamyl <0.5. Kelly, Shideman and Adams (5) observed results which were of the same

general order when the blood level at the time of return of righting reflexes was used as an index of potency, Surital having a value of 1 39 and Thioethamyl 0 57

TABLE IV

Comparative cumulative action of four thiobarbiturates as measured by increases in duration of anesthesia following administration of each compound in fixed dosage at hourly intervals

Increment in duration of anesthesia is expressed as percentage of initial anesthesia time

DRUG	DOSE (ACID WT)	NO OF DOGS	DURATION OF ANESTHESIA					
				Hour				
				0	1	2	3	4
	mgm / kgm							
Sodium thiopental	7 5	12	Minutes	7 6 ± 1 1	14 8 ± 2 0	31 5 ± 2 3	78 3 ± 8 1	
			Per cent of 0 hour time	100	195	414	1029 ± 106 (^{t}value = 9 6)	
Sodium Surital	5 0	16	Minutes	5 8 ± 1 6	6 5 ± 1 1	11 3 ± 1 5	22 5 ± 3 8	41 5 ± 9 3
			Per cent of 0 hour time	100	112	195	386 ± 64 (^{t}value = 6 0)	716
Sodium Thioethamyl	12 5	5	Minutes	2 4 ± 24	8 4 ± 1 2	28 8 ± 4 5	67 4 ± 9 7	
			Per cent of 0 hour time	100	350	1200	2808 ± 474 (^{t}value = 5 9)	
Sodium B 10	3 75	7	Minutes	6 3 ± 1 1	10 1 ± 2 4	22 6 ± 7 2	33 0 ± 5 7	45 5 ± 9 8
			Per cent of 0 hour time	100	160	358	523 ± 81 (^{t}value = 6 4)	722

TABLE V

Statistical significance of the differences in third hour durations of anesthesia on basis of comparison of per cent increases of the third hour values over the 0 hour values

DRUG	SOD PENTOTHAL	S SURITAL	S THIOETHAMYL	S.B 10
Sod Thiopental	—	5 16	3 65	3 77
Sod Surital	5 16*	—	5 06	1 32†
Sod Thioethamyl	3 65	5 06	—	4 74
Sod B 10	3 77	1 32†	4 74	—

* All values are ^{t}values of the differences between respective means

† The Sod B 10 and Sod Surital series' differences are not statistically significant

From an inspection of figure 1 and table IV it is quite apparent that Surital exhibits a lower rate of accumulation than either thiopental or Thioethamyl This is undoubtedly due to the greater inherent potency of Surital since as shown

by Kelly *et al.*, (5) the shape and slope of the blood level curves for all three drugs is similar indicating that the body detoxifies each compound at the same rate irrespective of its absolute potency as an anesthetic. The rate of cumulation is undoubtedly a function of the total dose administered, the more potent drugs requiring a smaller dose and hence a shorter time for detoxication. In addition, there may be degradation products which may either interfere with detoxication or possess a depressant action in their own right. Recently Shideman, Kelly and Adams (6), using the spectrophotometric method, followed blood levels

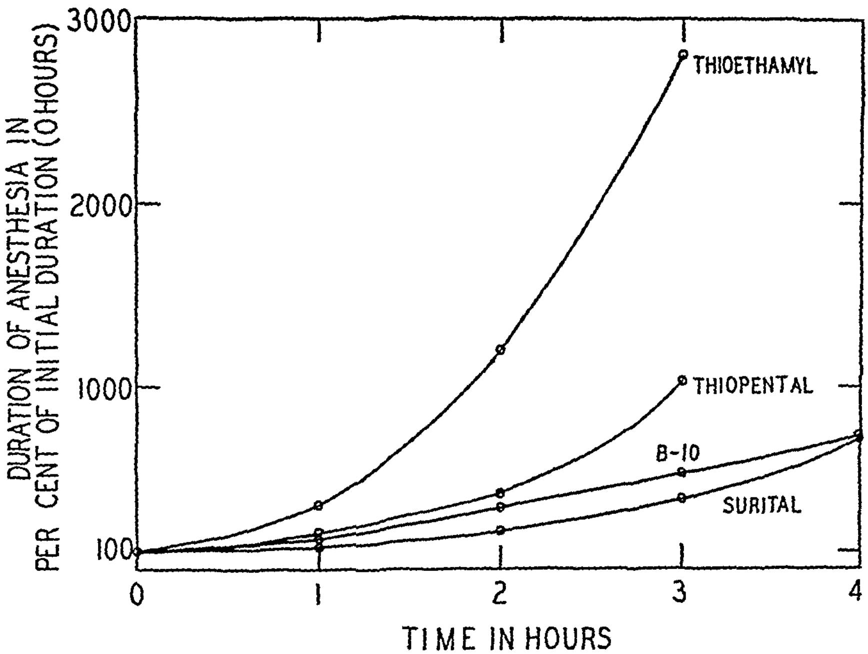


FIG. 1. CUMULATIVE ACTION OF HOURLY INJECTIONS

The time values on the abscissa represent the hour on which the injections were administered. Doses administered each hour are listed in table IV.

of thiopental in dogs receiving repeated doses of the drug at short intervals. The plasma level at which the righting reflexes returned was higher with each successive dose. This would indicate the development of an acute tolerance. However it is possible that degradation products, in addition to thiopental, were measured by the method and that as more and more breakdown products accumulated "apparent" higher values for thiopental were observed.

In conclusion it may be stated that Surital possesses certain advantages over some of the other more common intravenous anesthetic agents. It exhibits high potency, rapid induction and emergence with a few signs of stimulation, and produces satisfactory muscular relaxation. Respiratory depression is no greater than that observed with thiopental when both are given in equivalent anesthetic doses (3). The lower doses necessary with Surital should place less

strain upon detoxication mechanisms, allow for shorter post-operative sleeping time, and reduce the cumulative effect. Finally Surital shows low cardiac toxicity (3).

SUMMARY

The anesthetic properties of four thiobarbiturates, thiopental, Surital, Thioethamyl, and B-10, were compared in dogs. The quality of anesthesia was most satisfactory with Surital and thiopental. The ratio of potency, based upon the duration of anesthesia and using thiopental as a standard of 1.0, was Surital 1.5, Thioethamyl, <0.5 and B-10, >1.5.

Surital exhibited considerably less cumulative effect than either thiopental or Thioethamyl and possesses other characteristics noted above which indicate it to be worthy of clinical trial. B-10 showed cumulative action of much the same degree as Surital but because of undesirable side-reactions was not considered to be a good intravenous anesthetic agent for the dog.

Based upon these observations, and the assumption that all thiobarbiturates are detoxified in a similar manner, it seems clear that further work should center on a search for a more potent agent in order to permit a greater duration of anesthesia without serious cumulative effects.

REFERENCES

1. WYNGAARDEN, J. B., WOODS, L. A., AND SEEVERS, M. H.: Fed. Proc., 6: 388, 1947.
2. WYNGAARDEN, J. B., WOODS, L. A., RIDLEY, R., AND SEEVERS, M. H.: Fed. Proc., 6: 388, 1947.
3. WOODS, L. A., WYNGAARDEN, J. B., RENNICK, B., AND SEEVERS, M. H.: Fed. Proc., 6: 387, 1947; THIS JOURNAL, succeeding article.
4. BURSTEIN, C. L., AND ROVENSTINE, E. A.: THIS JOURNAL, 63: 42, 1938.
5. KELLY, A. R., SHIDEMAN, F. E., AND ADAMS, B. J.: Fed. Proc., 7: 233, 1948.
6. SHIDEMAN, F. E., KELLY, A. R., AND ADAMS, B. J.: Fed. Proc., 7: 255, 1948.

CARDIOVASCULAR TOXICITY OF THIOBARBITURATES: COMPARISON OF THIOPENTAL AND 5-ALLYL-5-(1-METHYLBUTYL)-2-THIOBARBITURATE (SURITAL) IN DOGS[1]

L. A. WOODS, J. B. WYNGAARDEN,[2] B. RENNICK, AND M. H. SEEVERS

Department of Pharmacology, University of Michigan, Ann Arbor

Received for publication November 10, 1948

Evidence and opinion is divided as to the primary cause of death from thiobarbiturates. It is well established, of course, that both thiobarbiturates and their oxygen analogues can produce respiratory failure. Certain investigators believe that thiopental (Pentothal) and other similar derivatives possess a primary cardiotoxic action sufficient to cause death. This belief is predicated upon evidence obtained from the dog as follows: (a) additive toxic effects with digitalis (1); (b) sudden fall in arterial pressure and appearance of ectopic beats (2); (c) conduction disturbances with cardiac arrythmia (3). Other investigators have been unable to establish a primary cardiotoxic action of thiobarbiturates. For example, Kohn and Lederer (4) found that thiopental did not produce primary cardiac death or ventricular fibrillation in the dog. They attributed all deaths to respiratory failure. Betlach, working with dogs (5), and Volpitto and Marangoni (6) in humans, found no significant changes in the electrocardiograms under thiopental anesthesia.

In view of the contradictory nature of previous studies, the experiments described herein (7) were designed to obtain if possible a definitive answer to this question by determining the direct cardiovascular toxicity of two thiobarbiturates by (a) means of the Starling heart-lung preparation, (b) comparison of lethal doses and types of death in animals spontaneously respiring with others respired artificially in order to control the factor of anoxia.

A. *Heart-Lung Preparation.* Ten experiments were performed using the Krayer-Mendez (8) modification of the Patterson-Starling heart-lung preparation, with the exception that the venous inflow pump and coronary artery cannula were not used. Also the Stolnikow stromuhr was employed instead of the Weese variety. The cardiac output and arterial resistance were adjusted at the outset to give a standard work value for all experiments.

Both thiopental and Surital were employed as the sodium salts in one per cent solution (acid weight). All injections were made into the superior vena cava. In a given experiment two and one-half cc. (25 mgm.) of the given thiobarbiturate were injected every fifteen minutes until failure ensued. Also, for purposes of direct comparison in the same preparation, in most experiments one (1) injection of the alternate thiobarbiturate was substituted for one of the drug being principally studied.

Continuous measurements of right auricular pressure, arterial pressure, and heart volume were recorded on the kymograph drum. Periodic measurements of the cardiac output (left ventricular output minus the coronary artery flow), and heart rate were recorded.

Increased right auricular pressure and decreased cardiac reserve were used as criteria of heart failure.

[1] Supported by a grant from Parke, Davis and Company, Detroit, Michigan.

[2] Present address: Department of Medicine, Massachusetts General Hospital.

B. *Intact Animal Experiments.* Twelve experiments were carried out in dogs to evaluate the response of the cardiovascular system to thiopental and Surital. In all preparations arterial pressure was recorded from the carotid artery, and the trachea was cannulated to facilitate respiration. Injections were made into the femoral vein. Electrocardiographic records were obtained in several experiments. The animals were anesthetized using a minimum amount of the particular thiobarbiturate to be studied.

Six of the twelve animals were studied under spontaneous respiration and in these abdominal and thoracic pneumographic tracings were recorded. The other six dogs were respired artifically with positive intratracheal insufflation.

Thiopental and Surital were used as the sodium salts both with intermittent injection at regular intervals and also continuous intravenous infusion. The drugs were used in amounts inversely proportional to their anesthetic potency (9), i.e., 1.5 times as much thiopental, by weight or concentration, as Surital.

RESULTS. A. *Direct Cardiac Effect.* (1) *Acute rise in right auricular pressure.* One of the actions of these drugs measurable in the heart-lung preparation is the rise in right auricular pressure immediately following the injection of the drug into the superior vena cava. This increase in pressure extends over a period of a few seconds to a few minutes. The right auricular pressure, following the acute rise, dropped but usually did not reach the pressure level before the injection. Occasionally with Surital, a fall below the initial level followed the acute rise in pressure. In one instance there resulted only a drop in auricular pressure. One injection of the alternate drug, Surital,was carried out in the five experiments of the thiopental series and one injection of the alternate drug, thiopental, was made in each of two of the experiments of the Surital series. For each series there is plotted in figure 1 the average acute right auricular pressure rise in mm. against the number of injections. The alternate drug is represented by a separate point. It was thus possible to compare responses of the same heart-lung preparation to each drug. Whether one injection of Surital is given in a series of thiopental injections or thiopental is given in a series of Surital injections, the acute right auricular pressure rise produced by Surital is less than that produced by thiopental, and occasionally Surital produced a fall in pressure on its first few injections.

(2) *Decreased cardiac reserve.* The normal heart-lung preparation responds to increased cardiac load (elevation of the venous blood reservoir) with increased cardiac output with the result that there occurs only a slight increase in right auricular pressure. The term cardiac reserve is frequently given to the capacity of the heart described above. Thus decreased cardiac reserve is measurable by recording the greater increase in right auricular pressure in response to elevation of the venous blood reservoir.

Right auricular pressure responses to increased cardiac load were analyzed in the following manner:

Index of cardiac reserve (ICR)

$$= \frac{\text{Elevation of reservoir (mm.)} - \text{Rt. auric. pressure rise (mm.)}}{\text{Elevation of reservoir (mm.)}} \times 100$$

The theoretical 100 per cent cardiac reserve would be present only if the elevation of the venous blood reservoir produced no rise of the right auricular pressure. Since such a rise always occurs, *the control ICR was equated to 100 per cent reserve for that particular preparation,* and subsequently determined ICR values were

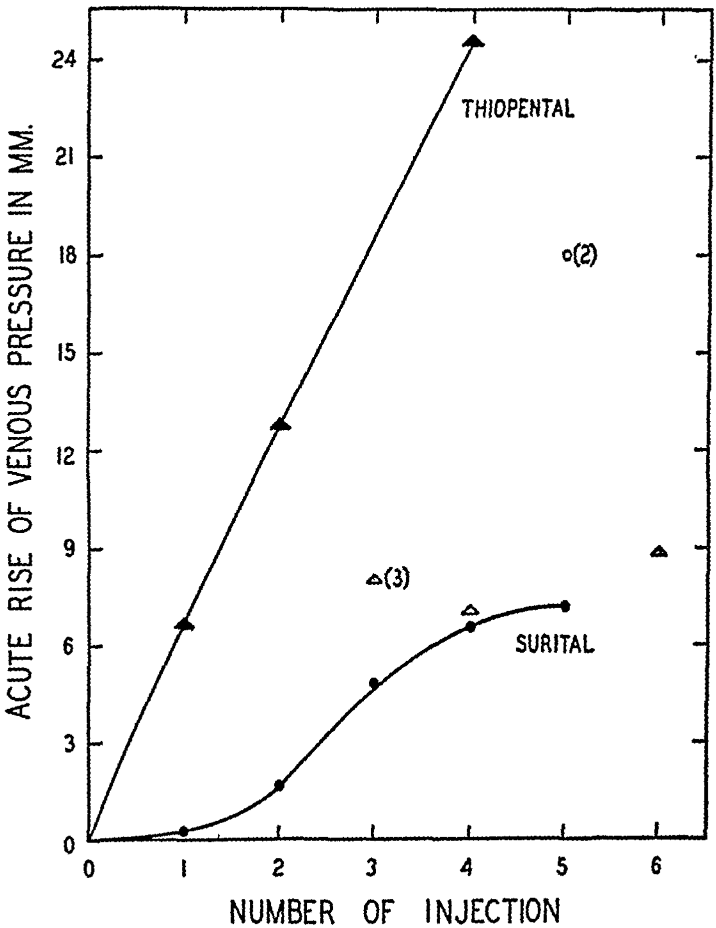


FIG. 1. ACUTE CARDIAC TOXICITY OF SURITAL AND THIOPENTAL

Points on the curves represent average immediate right auricular pressure responses to injections of the respective drug. The open triangles represent responses to substituted injections of Surital in the pentothal series. The open circle represents the responses to substituted injections of pentothal in the Surital series. The figures in parentheses represent the number of substituted injections whose average gave the point plotted. The points not thus marked represent one determination.

compared with this value in per cent of the initial control ICR figure. Thus, the per cent reserve equals (Determined ICR ÷ Control ICR) × 100. The theoretical number of injections (see table I) required to produce 50 per cent cardiac reserve in each heart-lung preparation was determined from figure 2, A and B. From the number of injections the weight in mgm. of the drug required to

TABLE I

Comparison of cardiotoxicities of thiopental and surital in heart-lung preparation*

DRUG	EXPER. NO.	NO. OF INJ. (25 MGM EA) REQUIRED TO PRODUCE 50% REDUCTION OF CARDIAC RESERVE	WEIGHT OF VENTRICLES (UNCORR)	DOSE AT 50% REDUCTION IN MGM/GRAM OF VENTRICLE	MEAN DOSE ±S.E m	t VALUE OF: Mean	t VALUE OF: Difference of means
			grams				
Surital	I	5.61	81.0	1.73			
	II	10.55	87.0	3.03			
	III	4.30	61.0	1.76	1.85 ±0.31	5.97	
	IV	4.52	76.5	1.48			
	V	4.90	95.2	1.29			1.11
Pentothal	I	5.60	83.9	1.67			
	II	6.05	81.0	1.86			
	III	3.30	87.0	0.94	1.47 ±0.16	9.19	
	IV	4.60	85.8	1.34			
	V	4.00	66.1	1.53			

* As determined by right auricular pressure rises with successive injections.

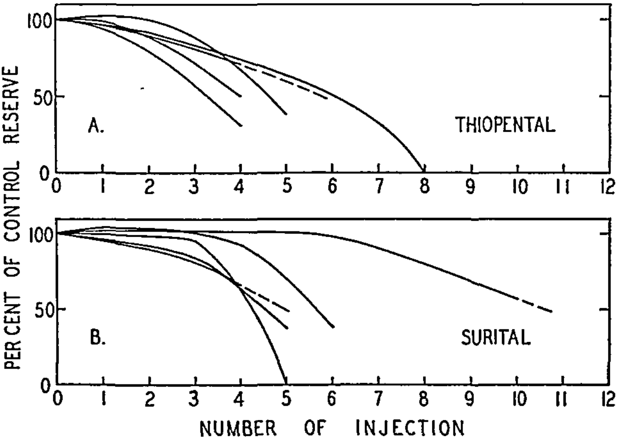


FIG. 2. RATES OF IMPAIRMENT OF CARDIAC RESERVE OF HEARTS UNDER SURITAL AND THIOPENTAL, ON BASIS OF RIGHT AURICULAR PRESSURE INCREASE

Each HLP response is plotted: in Figure 2A, the five HLPs given Sodium Thiopental, and in Figure 2B, the five HI [illegible]

might be obtained.

Those animals which were maintained under artificial respiration tolerated one and one-half to three and one-half times the amount of either Surital or thiopental that was tolerated without artificial respiration.

Discussion. Based upon observations in the heart-lung preparation Surital is perhaps less toxic, but certainly no more toxic in the absolute sense, than thiopental. This is in spite of the greater anesthetic potency of Surital, requiring only about two-thirds as much in quantity to produce a similar level of anesthesia. The ability of these two thiobarbiturates to produce cardiac failure is not markedly greater than with the oxygen analogues (11). Pentobarbital produces heart failure in the identical type heart-lung preparation in dosages comparable to those described herein.

It is of major interest to know whether death of the intact animal under thiobarbiturate is respiratory (anoxic) or cardiac (direct toxicity). From the accompanying tables and description of results it can be concluded that the animal under artificial respiration is capable of tolerating much larger amounts of the thiobarbiturates than under spontaneous respiration. Also the course of death is somewhat different. With spontaneous respiration there are frequent arrhythmias, and finally there results respiratory arrest followed by a sudden drop in blood pressure and cessation of heart beat. Under artificial respiration after a long period of normal pressure, the blood pressure slowly declines until shock levels are reached and death soon ensues. Abnormal electrocardiographic changes rarely occur except as a terminal event. This slow decrease in blood pressure may be due either to a peripheral vascular collapse resulting from direct drug toxicity or paralysis of the medullary vasomotor centers. Perhaps the former is the more important.

It is apparent, therefore, that respiratory failure is the primary cause of death in the intact dog. Most, if not all, of the cardiac abnormalities are due to a progressive anoxia resulting from inadequate respiratory exchange and may be avoided by insuring adequate oxygenation of the animal tissues. It is questionable whether direct cardiac toxicity plays any role whatever in intravenous human anesthesia with the thiobarbiturates.

SUMMARY

1. In the heart-lung preparation the thiobarbiturates, thiopental and Surital, exhibit only a moderate degree of cardiac toxicity which is no greater than that produced by the corresponding oxygen analogue. On an equal dose basis, Surital is perhaps less toxic, but definitely not more toxic, than thiopental.

2. In the intact dog, anoxia secondary to respiratory failure plays the predominant role in death from thiopental or Surital anesthesia. Abnormalities of cardiac rhythm appear to take origin on the basis of inadequate oxygenation.

3. Peripheral vascular failure, of anoxic or toxic origin, may be a contributory cause of death.

REFERENCES

1. Gruber, C. M., Haury, V. G., and Drake, M. D.: Am. Heart. J., **20**: 329, 1940.
2. Reynolds, C., and Veal, J. R.: S. Med. J., **31**: 650, 1938; Reynolds, C.: Anesth. and Analg., **18**: 270, 1939.

3. Gruber, C. M., Haury, V. G., and Gruber, C. M., Jr.: This Journal, 63: 193, 1938.
4. Kohn, R., and Lederer, L.: J. Lab. and Clin. Med., 23: 717, 1938.
5. Betlach, C. J.: This Journal, 61: 329, 1937.
6. Volpitto, P. P., and Marangoni, B. A.: J. Lab. and Clin. Med., 23: 575, 1938.
7. Woods, L. A., Wyngaarden, J. B., Rennick, B., and Seevers, M. H.: Fed. Proc., 6: 387, 1947.
8. Krayer, O., and Mendez, R.: This Journal, 74: 350, 1942.
9. Wyngaarden, J. B., Woods, L. A., Ridley, R., and Seevers, M. H.: This Journal, preceding article.
10. Wood, E. A., and Moe, G. K.: Am. J. Physiol., 136: 515, 1942.
11. Moe, G. K.: Personal communication.

THE CHEMOTHERAPEUTIC AND PHARMACOLOGICAL PROPERTIES OF THE L-EPHEDRINE SALT OF PENICILLIN G (TERSAVIN[1])

E. GRUNBERG, L. O. RANDALL, AND R. J. SCHNITZER

Chemotherapy and Pharmacology Laboratories, Research Department, Hoffman-La Roche Inc., Nutley, New Jersey

Received for publication November 10, 1948

Mixtures of penicillin with vasoconstrictors such as epinephrine (Fisk and coworkers (1), Fisken *et al.* (2), Ercoli *et al.* (3) and Schachter (4)) have been used experimentally and clinically in order to obtain delayed absorption as indicated by prolonged blood levels.

It is not a rare occurrence in the field of chemotherapy and pharmacology that the characteristic effects of biologically active agents disappear if the constituents are combined in one molecule as a chemical entity even though their mixture shows the expected activity. For that reason, it appeared desirable to study the properties of an l-ephedrine salt of penicillin G which was recently prepared by Dr. M. W. Goldberg and Mr. S. Teitel in the Roche Chemical Laboratories.

The l-ephedrine salt of penicillin G (Tersavin) is a new penicillin derivative which combines crystalline penicillin G and l-ephedrine. Tersavin, which has the empirical formula: $C_{16}H_{18}O_4N_2S \cdot C_{10}H_{15}ON$, is a crystalline white powder with a melting range of 135–137°C. (decomp.). The specific optical rotation $[\alpha]_D^{20°}$ in a 2.4 per cent aqueous solution is +190°. The substance is highly soluble in water (60 per cent at room temperature) and a 1.5 per cent aqueous solution has a pH of 6.2. The calculated unitage per 1 mgm. is 1187 units; this was confirmed by assay. Therefore, the potency of 1.4 mgm. of Tersavin corresponds to that of 1 mgm. of crystalline sodium penicillin G.

The present paper consists of the results of the toxicological, pharmacological and chemotherapeutic work carried out with this compound.

PART I. *Pharmacological Properties of Tersavin*

Tersavin and ephedrine HCl have been studied for their comparative toxicity, circulatory effects and bronchodilator action.

Toxicity. The toxicity values for Tersavin and ephedrine HCl were determined in mice, rats and rabbits by various routes of administration. The LD_{50} toxicity values and their standard errors were calculated by a graphic method (5). Since the molecular weight of Tersavin (499.6) is 2.48 times that of ephedrine HCl (201.7), the data are given in terms of mgm./kgm. and millimols/kgm.; the latter shows the true relative toxicities. The data in table 1 indicate that Tersavin is only slightly more toxic than ephedrine HCl in mice and rats but about equally toxic in rabbits. The toxicity of Tersavin may, therefore, be ascribed to its ephedrine content.

[1] Tersavin—T. M.—Reg. U. S. Pat. Off.

Circulatory Effects The relative blood pressure effects of Tersavin and ephedrine HCl were each measured in 8 dogs and 4 cats The animals were anesthetized with dial-urethane, the drugs were administered intravenously and the blood pressure recorded with a mercury manometer The animals were atropinized (1 mgm /kgm) and responses to a series of graded doses of epinephrine were measured A dose of Tersavin or ephedrine HCl was given and from the response in mm Hg the relative potency in terms of epinephrine was estimated A dose of Tersavin was followed by an equimolar dose of ephedrine HCl, and vice versa, in order to observe the tachyphylactic effects Typical records from 2 dogs are shown in figure 1 This illustrates that the vasoconstrictor potencies of Tersavin and ephedrine HCl are identical on a molecular basis and each compound produces tachyphylaxis to the other Although there is wide variation in sensitivity of animals to both Tersavin and ephedrine HCl as well as to epinephrine, the ephedrine salts are approximately 1/200 as strong as epinephrine in dogs and 1/100 as strong in cats

TABLE 1

Toxicity of Tersavin and ephedrine HCl

SPECIES	ROUTE	EPHEDRINE HCl $LD_{50} \pm$ S E		TERSAVIN $LD_{50} \pm$ S E	
		mgm /kgm	*m mol/kgm*	*mgm /kgm*	*m mol/kgm*
Mice	i p	340 ±51	1 69 ± 25	630 ±69	1 26 ± 14
Mice	i v	95 ±19	47 ± 09	175 ±17	35 ± 03
Rats	i p	290 ±20	1 44 ± 10	680 ±88	1 36 ± 18
Rats	s c	1150	5 82	2400	4 80
Rabbits	i v	65	32	175	35

Bronchodilator Action The relative potency of Tersavin and ephedrine HCl as bronchodilators in comparison with epinephrine was measured on the isolated tracheal smooth muscle of guinea pigs by the method of Castillo and de Beer (6) A chain of isolated tracheal rings, attached to a light lever, was suspended in Hastings Van Dyke solution, aerated with 95 per cent O_2: 5 per cent CO_2 and kymographic tracings of the changes in tone were recorded The response to graded doses of epinephrine was obtained and a dose of Tersavin or ephedrine HCl was then added

From the degree of relaxation, the potency of the ephedrine salts were estimated in terms of epinephrine Since ephedrine salts could be washed out only with great difficulty, successive doses of the compound usually had less effect than the first so that graded responses could not be obtained Figure 2 illustrates the bronchodilator action of Tersavin and ephedrine on different preparations From 6 experiments with each compound an average potency of 1/500 of epinephrine was found The effects of Tersavin were indistinguishable from those of equimolar concentrations of ephedrine HCl

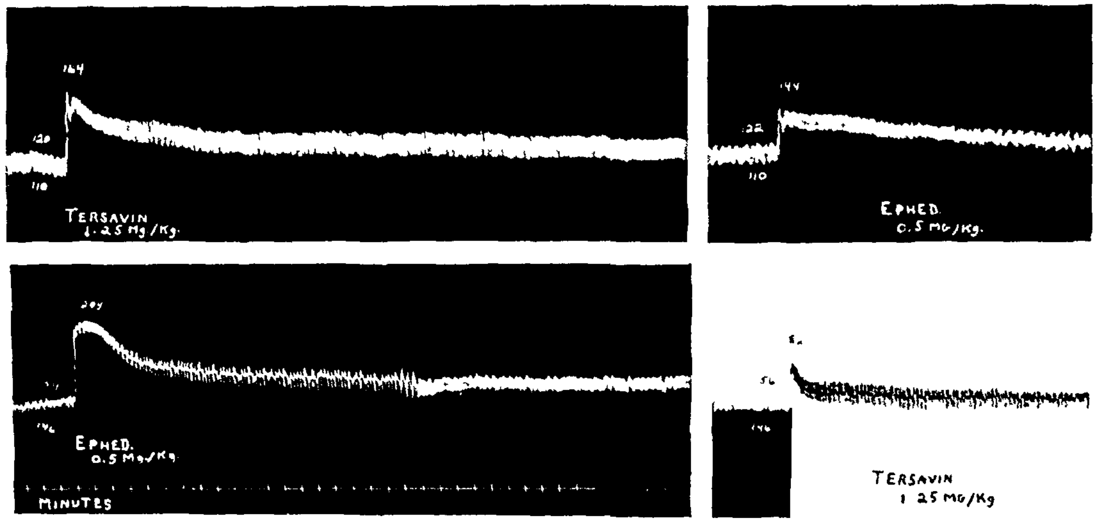


FIG. 1. CIRCULATORY EFFECTS OF TERSAVIN AND EPHEDRINE HCl

			micromoles
A.	Epinephrine .	2 μ/kgm.	= 011
	Epinephrine	4 μ/kgm	= 022
	Tersavin	1 25 mgm./kgm.	= 2 5
	Ephedrine HCl	0 5 mgm /kgm.	= 2.5
B.	Epinephrine	2 μ/kgm	= 011
	Epinephrine	4 μ/kgm.	= 022
	Ephedrine HCl	0 5 mgm /kgm.	= 2.5
	Tersavin	1 25 mgm /kgm.	= 2 5

Time in minutes. Numbers on blood pressure records indicate mm. Hg.

Part II *Chemotherapeutic Properties of Tersavin*

Materials and methods *Penicillins* The sodium salt of crystalline penicillin G (1667 units/mgm) and the l ephedrine salt were obtained from the Roche Chemical Laboratories

Strains of Test Organisms The origin of the strains used in this investigation have been described in previous publications by Schnitzer *et al* (7) The following bacterial strains were used

(1) *In vitro experiments*

β hemolytic streptococci (Group A)

Strept 4, type 3

Strept C203, type 3

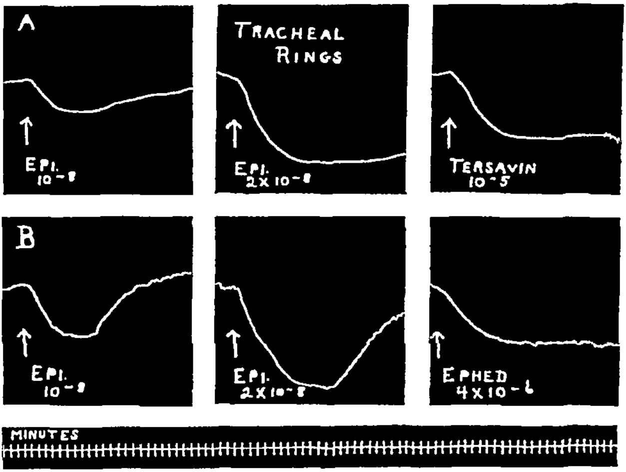


Fig 2 Action on Guinea Pig's Tracheal Rings

A Epinephrine, 10^{-8} gm /cc = 0 055 micromolar
Epinephrine, 2×10^{-8} gm /cc = 0 11 micromolar
Tersavin, 10^{-5} gm /cc = 20 micromolar

B Epinephrine, 10^{-8} gm /cc = 0 055 micromolar
Epinephrine 2×10^{-8} gm /cc = 0 11 micromolar
Ephedrine HCl 4×10^{-6} gm /cc = 20 micromolar

A and B are records from different preparations Time in minutes

Strept B—from an abscess of the foot

Strept 98—source unknown

Staphylococcus aureus

Staph 209

Staph L

Staph 13—from nose culture

Staph 49—from tonsillar culture

Pneumococci

Pn 6301, Type I

Pn 6302, type II

Pn 6303, type III

Pseudomonas pyocyaneus
Strain [illegible] from nose culture
Strain 17 from dog's ear culture
Organisms of the coli typhoid group
E. coli I
E. coli III McLeod strain
E. typhosa I
E. typhosa P58A
S. schottmuelleri 10

(2) *In vivo experiments*
β hemolytic streptococci (Group A)
Strept. 4, type 3
Strept. B
Pneumococci
Pn. 6301, type I
Spirochetes
Borrelia Novyi

Technique of the in vitro Tests. Serial dilutions of sodium penicillin G and Tersavin were made in the semi-synthetic medium of Adams and Roe (8) in the case of streptococci and pneumococci and in papain digest bacto-beef broth in the case of all other organisms. The tubes were immediately inoculated with one drop of the culture dilution (streptococci—undiluted; pneumococci—undiluted; pseudomonas—10^{-5}; coli-typhoid group—10^{-7}) from a [illegible] pipette. The test tube racks were then shaken, placed in an incubator at 37°C. and read after 24 hours.

Technique of the in vivo Tests. Eighteen–twenty gram mice, taken from one breeding colony, were used in all the experiments.

1. Systemic infections with streptococci and pneumococci. (a) Streptococci. Mice were injected intraperitoneally with 0.5 cc. of a 10^{-5} dilution of a 22-hour serum broth culture of Streptococcus hemolyticus #4 (Group A, type 3). This dose corresponds to 1000 MLD.

The treatment consisted of variations of our standard total dose of penicillin, 500 units/kgm. (0.3 mgm./kgm. crystalline penicillin G), as described by Soo-Hoo and Schnitzer (9). The compounds were given in 2 treatments, one immediately following infection and the other 4 hours later.

(b) Pneumococci. Mice were injected intraperitoneally with 0.3 cc. of a 22-hour serum broth culture of Pneumococcus 6301 (type I). This dose represents about 1000 MLD of the strain.

The treatment dose as described by Kelly and Schnitzer (10) was used. It consisted in the injection of a total of 1500 units/kgm. (0.9 mgm./kgm. crystalline penicillin G) and 3000 units/kgm. (1.8 mgm./kgm. crystalline penicillin G) in 2 treatments. The first dose was given immediately after infection while the second was given 4 hours later.

The animals in both the streptococcal and pneumococcal experiments were observed for a 3 week period. All mice that died during this time were autopsied and cultures of the heart blood were made.

(2) Systemic infection with Borrelia Novyi. The compounds were evaluated by the method described by Buck, Farr and Schnitzer (11). It consisted of the treatment of a fully developed borrelia infection in mice with a single standard dose of penicillin G, 25,000 units/kgm. (15.0 mgm./kgm. crystalline penicillin G). The number of spirochetes in 100 fields of the darkfield microscope was counted before the treatment and 3 and 20 hours after the treatment. From these counts, the reduction in the number of parasites in the blood was determined.

(3) Local infection. The technique of the local infection and its treatment has been previously described by us (12).

(a) Local therapeutic experiments Two tenths cc of a 1 10[3] dilution of a 22 hour serum broth culture of β hemolytic streptococcus B were injected into the ventral subcutaneous tissue of mice The animals were treated immediately, by subcutaneous injection into the infected area, with 1 0 cc of a solution of the penicillin salts ranging from 2 5 to 20 units/cc (0 0015–0 012 mgm /cc crystalline penicillin G) At the end of 22–24 hours the animals were autopsied and cultures from the infected and treated areas were made on blood agar plates Animals from which cultures showed less than 10 colonies are listed as successfully treated in the tables

(b) Local prophylactic experiments One cc of a solution of the penicillin salts containing 250 or 500 units/cc (0 15 or 0 3 mgm /cc crystalline penicillin G) was injected into the ventral subcutaneous tissue of mice At specified intervals of 15 minutes, 30 minutes, 1 hour and 2 hours after injection of the penicillin salts, 0 2 cc of a 1 5 dilution of a 22 hour

TABLE 2

Comparative bacteriostatic activity of Tersavin and penicillin G in vitro

ORGANISM	BACTERIOSTATIC CONCENTRATION			
	Tersavin		Penicillin G	
	units/cc	*mgm /cc*	*units/cc*	*mgm /cc*
Streptococcus 4	0 0195	0 000016	0 0195	0 000012
Streptococcus C 203	0 0195	0 000016	0 0098	0 000006
Streptococcus B	0 0195	0 000016	0 0098	0 000006
Streptococcus 98	2 5	0 0021	2 5	0 0015
Staphylococcus 209	0 0195	0 000016	0 0195	0 000012
Staphylococcus L	>10 0	>0 0084	>10 0	>0 006
Staphylococcus 13	0 039	0 000033	0 039	0 000023
Staphylococcus 49	0 625	0 00053	0 625	0 00037
Pneumococcus 6301	0 0195	0 000016	0 0195	0 000012
Pneumococcus 6302	0 0195	0 000016	0 0098	0 000006
Pneumococcus 6303	0 0195	0 000016	0 0195	0 000012
Pseudomonas 35	>100 0	>0 084	>100 0	>0 06
Pseudomonas 45	>100 0	>0 084	>100 0	>0 06
E coli J	25 0	0 021	12 5	0 0075
E coli 119	50 0	0 042	25 0	0 015
E typhosa F	25 0	0 021	12 5	0 0075
E typhosa P 58 A	12 5	0 01	6 25	0 0037
S schottmuelleri 10	50 0	0 042	25 0	0 015

serum broth culture of Streptococcus B were injected subcutaneously into the treated area At the end of 22–24 hours, the mice were autopsied and the area of infection or infection and treatment, respectively, was swabbed Cultures from these swabs were then made on blood agar plates

In both types of experiments, it was unnecessary to use blood agar plates into which a penicillin inhibitor was incorporated for making cultures from the animals, since we had found that all the penicillin had disappeared much earlier (12) and there was no danger of carrying over enough penicillin to cause any bacteriostatic activity

[3] A dilution of 1 10 was used in the local therapeutic experiments, since it was found that at the time these experiments were run, a dilution 1 10 gave the same response as had a dilution of 1 5

EXPERIMENTAL. *Chemotherapeutic Activity in vitro.* Eighteen strains of gram positive and gram negative bacteria were tested, by the dilution method, for their sensitivity to Tersavin and crystalline penicillin G. As table 2 shows, representative organisms of the group of penicillin sensitive and penicillin insensitive organisms were selected for these tests. One naturally resistant organism was included among the penicillin sensitive strains of staphylococci. It is evident that the bacteriostatic activity of Tersavin is identical with that of penicillin G. All differences are not greater than one dilution step and this difference is

TABLE 3

Activity of Tersavin and penicillin G in the systemic streptococcus 4 infection of mice

TOTAL DOSE	TOTAL DOSE	NO. OF MICE	SURVIVORS	CD_{50} ± S.E.	CD_{50} ± S.E.
units/kgm.	*mgm./kgm.*		*per cent*	*units/kgm.*	*mgm./kgm.*
Tersavin					
1000	0.84	29	100		
500	0.42	30	88.3	220 ±53*	0.185 ±0.045
250	0.21	30	58.3		
Penicillin G					
1000	0.6	30	98.4		
500	0.3	30	85.0	308 ±52	0.185 ±0.031
250	0.15	30	35.0		
Controls	—	30	0		

* According to the method described by De Beer (4).

TABLE 4

Activity of Tersavin and penicillin G in the type I pneumococcus infection of mice

TOTAL DOSE	TOTAL DOSE	NO. OF MICE	SURVIVORS	CD_{50} ± S.E.	CD_{50} ± S.E.
units/kgm.	*mgm./kgm.*		*per cent*	*units/kgm.*	*mgm./kgm.*
Tersavin					
3000	2.52	10	100		
1500	1.26	20	65	1300 ±312	1.07 ±0.26
Penicillin G					
3000	1.8	10	100		
1500	0.9	20	45	1515 ±288	0.91 ±0.17
Controls	—	30	0	—	—

not significant since any dilution test is only accurate to plus or minus one dilution.

Systemic Activity in vivo. (1) Anti-streptococcal activity. The results of the experiments in the streptococcal infection are given in table 3. The total dosage is based on 2 treatments given on the day of the infection at 4-hour intervals. It is evident that the effect of Tersavin is identical with that of penicillin G, the differences in the unitage of the CD_{50} being insignificant.

(2) Anti-pneumococcal activity. The comparison of Tersavin and penicillin G in the pneumococcal infection, as given in table 4, shows that the standard

dose of 3000 units/kgm prevented the death of all infected mice when it was given in the form of the sodium salt (1 8 mgm /kgm) or in the form of Tersavin (2 52 mgm /kgm) The effect of half this dose was also about the same with both drugs and the CD_{50} on the basis of unitage exhibited, therefore, no significant difference The lower content of crystalline penicillin G made, of course, the CD_{50} by weight appear slightly better

(3) Anti-borrelia activity If tested by the rapid assay method with Borrelia Novyi (11), the activity of Tersavin was unit for unit the same as that of penicillin G (table 5) On a weight basis, about 30 per cent more Tersavin was required, which corresponds to the lower penicillin content of this salt

If the activity of Tersavin was evaluated in a therapeutic experiment, that is by repeated treatment of a manifest blood infection, the effect was also identical with that of penicillin G In this type of experiment, the mice received 8 subcutaneous treatments, 4 per day at 2 hour intervals, of 25,000 or 50,000 units/kgm (15 or 30 mgm crystalline penicillin G/kgm or 21 or 42 mgm Tersavin/

TABLE 5

Rapid assay of Tersavin and penicillin G in the Borrelia novyi infection of mice

Initial count 468 ±42 parasites/100 microscopic fields Single subcutaneous treatment

COMPOUND	DOSE	DOSE	NO OF TESTS	REDUCTION OF PARASITES		RATIO t 3/t 20
				t 3[a]	t 20	
	units/kgm	*mgm /kgm*		*per cent*	*per cent*	
Tersavin	25,000	21 0	2	99 3	93 2	1 08
Penicillin G	25,000	15 0	3	99 5	92 3	1 07
Controls	—	—	3	65 incr[b]	500 incr	—

[a] t 3 = count after 3 hours, t 20 = count after 20 hours

[b] incr = % increase of spirochetal count

kgm) of the penicillin salts to a total of 200,000 or 400,000 units/kgm respectively, (120 or 240 mgm crystalline penicillin G/kgm or 168 or 336 mgm Ter savin/kgm) beginning 1 day after the intraperitoneal injection of 400,000 to 500,000 parasites of Borrelia Novyi The mice treated with the higher dose of the penicillin salts stayed free of relapses for a period of 3 weeks Sixty per cent of the mice treated with the lower dose relapsed 10–14 days after the termination of therapy

It did not seem surprising that the activity of Tersavin was not significantly different from that of penicillin G in the treatment of generalized infections We have found in acute infections of mice that penicillin preparations showing a slow rate of absorption, induced by different vehicles or by the use of penicillin derivatives of lower solubility in water, exert the same activity as the water soluble salts of penicillin G A similar conclusion can also be drawn from results observed by Hobby (13, 14) The anti pneumococcal and anti streptococcal activity of procaine penicillin of low solubility which was administered in 1 dose was about the same as that of penicillin G administered in 3 doses It would

seem, therefore, that in the control of the acute fatal coccal infections of mice sufficient penicillin is released even if the absorption has been delayed by one means or another.

It might seem appropriate to point out that the reduction of the penicillin administration to not more than 2 treatments has been practiced in this laboratory for a long time. It is based on the early experience that in an experimental infection of mice the subdivision into small multiple doses does not offer an advantage over 1 to 2 larger doses. Similar observations have recently been published by Marshall (15), Zubrod (16), White *et al.* (17) and Gibson (18).

Blood level determinations in rabbits. Albino rabbits, weighing 2–3 kgm., were injected with 150,000 units (90 mgm. G; 126.4 mgm. Tersavin) dissolved in saline. Four animals were included in each group and the blood level was determined at the end of 1, 3, 5, 7 and 16 hours. At the 24-hour interval only 2 animals were used for each compound.

The bacteriostatic activity of the serum was determined in a dilution test with a β-hemolytic streptococcus (Strain #4) as the test organism. In the semi-synthetic media of Adams and Roe, used for the experiments, this strain was inhibited by 0.005–0.01 units (0.000003–0.000006 mgm.) penicillin G.

In all instances where 0.4 cc. serum or more was used in order to show growth inhibition of the organism, parallel experiments were carried out in tubes containing penicillinase. This was done in order to show that the bacteriostatic activity of the serum was actually due to penicillin.

The bacteriostatic concentrations of the serum after administration of penicillin G followed the familiar pattern. Expressed in penicillin units per cc., a peak of bacteriostatic activity was observed after 1 hour (average value: 38.4 units/cc.). This value dropped considerably after 3 hours when only 0.3 units/cc. were found. Only 1 animal out of 4 showed a value of 0.05 units/cc. after 5 hours. At later intervals no activity of the serum was observed.

The l-ephedrine salt of penicillin G (Tersavin), tested under the same conditions as above, gave similar but not identical results. The average peak concentration after 1 hour was found to be 51.2 units/cc. After 3 hours the average concentration was 18.4 units/cc., and even after 5 hours an average figure of 2.5 units/cc. was found. No demonstrable blood level was observed after 7 hours and at the later intervals.

It might be mentioned that the comparatively high value for Tersavin at the 5-hour interval was influenced by the fact that 1 rabbit exhibited a blood level of 6.4 units/cc. Even if this result were omitted from the data, the average blood level would still be 1.2 units/cc. which is considerably higher at this interval than that obtained after the administration of penicillin G.

These observations indicate that the presence of the vasoconstrictor did indeed delay the absorption of the ephedrine salt for at least 2 hours.

Differences, comparable to those observed above, may also be seen if one studies the local prophylactic anti-bacterial effect of penicillin salts. Even slight delays in absorption, such as may be induced by suspensions, have an influence which can be determined by the fate of a topical infection at the site of previous drug administration.

Topical activity in vivo. As described earlier in this paper, the technique

consisted in the subcutaneous infection of mice with β-hemolytic streptococci at a site where they had been treated previously with penicillin G or Tersavin. The mice were sacrificed at the end of 22–24 hours and cultures, from the treated and infected areas, were made on blood agar plates. The plates were read after 24-hours incubation.

If an interval of 15 or 30 minutes was kept between the prophylactic treatment and the infection, no significant difference was seen at the two doses in the percentage of mice successfully treated (table 6). After a 1-hour interval, differences became apparent and the number of mice in which the tissues were successfully treated was markedly higher after the administration of Tersavin than in the case of penicillin G. No significant difference was observed if 2 hours were allowed to elapse even though the percentage of successfully treated mice due to Tersavin treatment was slightly higher.

TABLE 6

Local prophylactic activity of Tersavin and penicillin G at various time intervals

COMPOUND	UNITS PER CC.	MGM. PER CC.	PER CENT MICE SUCCESSFULLY TREATED				
			15 min.*	30 min.	1 hr.	2 hrs.	Controls
Tersavin	500	.42	86†	82	57‡	22	0
Penicillin G	500	.3	73	59	23‡	0	0
Tersavin	250	.21	68	64	68§	22	0
Penicillin G	250	.15	50	64	27§	17	0

* Time of infection after treatment.

† The figures represent the results obtained with 10–30 mice for a group. Mice were considered successfully treated if the subculture on a blood agar plate showed less than 10 colonies.

‡ Significant figures because $p = 0.05$.

§ Significant figures because $p = 0.01$.

These observations seem to indicate that there is a definite tendency to prolonged prophylactic activity on the part of Tersavin.

If the technique of these experiments was changed so that the infection preceded the treatment which was administered shortly afterward, the mice were found to be successfully treated on the administration of 10–20 units penicillin G or Tersavin. This dose has been found, in many previous experiments, to be the normal dose for successful treatment of the animals. In a few instances where penicillin was more active, Tersavin was also more active.

Discussion. From the work presented in the experimental parts of this paper, the conclusion may be drawn that the l-ephedrine salt of penicillin G, Tersavin, exerted the specific activity of ephedrine as well as the characteristic anti-bacterial properties of penicillin G. There does not seem to be either an antagonism or a synergism between the components since the systemic activity of l-ephedrine penicillin did not seem to be significantly increased by the vasoconstrictor. Two modifications might, however, be attributed to the presence of the vasoconstrictor. One is an influence on the blood level which is prolonged and higher

with Tersavin than in the case of penicillin G; the other is an extended prophylactic topical effect in an experimental local streptococcal infection.

We feel, therefore, that l-ephedrine penicillin, Tersavin, offers the advantages of both the antibacterial properties of penicillin G and the vasoconstrictory effect of ephedrine by the administration of a single chemically defined compound.[3]

SUMMARY

Experimental data are submitted which show that the l-ephedrine salt of penicillin G, Tersavin, exerts quantitatively the anti-bacterial effect of penicillin G and the vasoconstrictory properties of l-ephedrine.

ACKNOWLEDGEMENT. The authors wish to express their gratitude to the technical staff of the laboratories, particularly Miss E. Titsworth, Mrs. D. Eldridge, Miss C. Unger, Miss A. Farr, Mrs. L. Lock and Mr. W. G. Peterson, who cooperated in carrying out the experiments.

REFERENCES

1. FISK, R. T., FOORD, A. G., AND ALLES, G.: Science, **101**: 124, 1945.
2. FISKEN, R. A., AND GRUHZIT, O. M.: Am. J. Syphilis, **30**: 581, 1946.
3. ERCOLI, N., HUEPER, W. C., LANDIS, L., LEWIS, M. N., AND SCHWARTZ, B. S.: Am. J. Med. Sci., **215**: 498, 1945.
4. SCHACHTER, R. J.: Proc. Soc. Exper. Biol. and Med., **68**: 29, 1948.
5. DE BEER, E. J.: THIS JOURNAL, **85**: 1, 1945.
6. CASTILLO, J. C., AND DE BEER, E. J.: THIS JOURNAL, **90**: 104, 1947.
7. SCHNITZER, R. J., FOSTER, R. H. K., ERCOLI, N., SOO-HOO, G., MANGIERI, C. N., AND ROE, M. D.: THIS JOURNAL, **88**: 47, 1946.
8. ADAMS, M. H., AND ROE, A. S.: J. Bact., **49**: 401, 1945.
9. SOO-HOO, G., AND SCHNITZER, R. J.: Arch. Biochem., **5**: 99, 1944.
10. KELLY, D. R., AND SCHNITZER, R. J.: Arch. Biochem., **7**: 461, 1945.
11. BUCK, M., FARR, A. C., AND SCHNITZER, R. J.: Science, **104**: 370, 1946.
12. GRUNBERG, E., SCHNITZER, R. J., AND UNGER, C.: Yale J. Biol. and Med., **20**: 479, 1948.
13. HOBBY, G. L., BROWN, E., AND PATELSKI, R. A.: Proc. Soc. Exper. Biol. and Med., **67**: 7, 1948.
14. HOBBY, G. L., LENERT, T. F., AND HYMAN, B.: J. Bact., **54**: 305, 1947.
15. MARSHALL, E. K., JR.: Meeting Antibiotic Study Sec., Nat. Inst. of Health, Wash., D. C., Oct. 1, 1947.
16. ZUBROD, C. G.: Bull. Johns Hopkins Hosp., **81**: 400, 1947.
17. WHITE, H. J., BAKER, M. J., AND JACKSON, E. R.: Proc. Soc. Exper. Biol. and Med., **67**: 199, 1948.
18. GIBSON, C. D., JR.: Proc. Soc. Exper. Biol. and Med., **67**: 278, 1948.

[3] Experiments which show the effect of Tersavin on the pathological and normal flora of the nasal mucosa will be reported elsewhere.

ANTAGONISTS FOR FATAL AND NON-FATAL DOSES OF QUININE INTRAVENOUSLY IN DEPRESSED CIRCULATORY STATES AND IN HYPERTHERMIA[1]

ROBERT H. DREISBACH

Department of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco 15, California

Received for publication November 12, 1948

Although new anti-malarial agents are coming into widespread use, quinine will probably continue to be used, especially when intravenous use is indicated in severe falciparum malaria with coma or other cerebral symptoms (1). However, intravenous injection of the alkaloid might be hazardous, especially in preexisting circulatory depression. Since this matter has not been previously explored, it was decided to obtain evidence on the toxicity of the alkaloid, and on the value of certain antagonists for its circulatory depression, in hemorrhage, asphyxia, hyperthermia, and shock. This paper presents the results obtained.

METHODS. The same general methods were followed as in a previous report (2). In addition to comparisons of the depressor effect of single doses (10 mgm./kgm.) of quinine in 0.9 per cent sodium chloride solution injected in 2 minutes, and the same dose of quinine mixed with an antagonistic agent, it was thought desirable that the most promising agent, epinephrine, should also be studied for possible antagonism to the fatal dose of quinine. For this purpose, the fatal dose of quinine hydrochloride was determined by continuous injection of a 1 per cent solution in normal saline solution at the rate of 2 mgm./kgm. per minute. This was then compared with the fatal dose of quinine when mixed with epinephrine in the ratio of 0.004 mgm. to 2 mgm. of quinine.

Both rabbits and cats were used. Anesthesia was produced with pentobarbital sodium, 35 mgm./kgm. being used intraperitoneally in cats, and intravenously, in rabbits. The various abnormal states were produced as follows: hemorrhagic shock by bleeding animals from the right femoral artery (blood pressures: 50–60 mm. Hg, rabbits and 70–80 mm. Hg, cats); moderate asphyxia with cyanosis and no change in blood pressure by allowing the animal to breathe through a 4-foot length of ¼ inch rubber tubing, or by clamping partially the trachea; severe asphyxia with variable though moderate blood pressure changes by using a 6-foot tube; shock with hemorrhage by opening the abdomen and gently manipulating and tugging on the mesenteries and viscera (blood pressures: 50–60 mm. Hg, rabbits and 90–100 mm. Hg, cats). For hyperthermia the body temperature of the anesthetized animals was raised to 41°–42°C. by tying the animal supine on an electrically heated pad. Although small numbers of animals were used in each state, due to non-availability, differences in effects were significant in most cases.

Small Doses of Quinine and Antagonists in Various Abnormal States (table 1). Epinephrine (0.015 mgm./kgm.) was the only drug which consistently antagonized the blood pressure lowering effect of quinine (10 mgm./kgm.) and increased recovery of blood pressure in the various abnormal states studied. After hemorrhage in rabbits, epinephrine decreased the blood pressure fall from a control of

[1] Supported, in part, by a grant from the Therapeutic Research Committee of the Council on Pharmacy and Chemistry of the American Medical Association.

hemorrhage in cats. In moderate or severe asphyxia in either cats or rabbits, epinephrine was not effective as an antagonistic agent. In non-hemorrhagic shock the median fatal dose for quinine alone, in rabbits, was 90 mgm./kgm.; in cats, 71 mgm./kgm. After epinephrine was added to the quinine, the median fatal dose was 208 mgm./kgm. in rabbits, 99 mgm./kgm. in cats. In hyperthermia, the median fatal dose for quinine was 50 mgm./kgm. in rabbits, and 56 mgm./kgm. in cats. Addition of epinephrine raised the median fatal dose of quinine to 150 mgm./kgm. in rabbits, 130 mgm./kgm. in cats.

Discussion. In the various abnormal states studied, namely, non-hemorrhagic shock, hemorrhage, asphyxia, and hyperthermia, the toxicity and depressor effect of quinine were almost uniformly increased. Any of these conditions might be found in clinical malaria, either as a result of the disease process itself, or of injury, or other diseases. High fever, of course, is almost always present in severe malaria, and this was found to increase the toxicity of quinine. Epinephrine, however, was quite effective in raising the fatal dose, and in antagonizing the depressor effect of quinine, thus suggesting its use in clinical malaria with high fever when quinine must be given intravenously, and assuming a similar antagonism.

Partial asphyxia in malaria might easily result from the decreased oxygen carrying capacity of the blood due to the clumping or sludging of red blood cells, or to concomitant pneumonia, or other disease process. While moderate asphyxia seemed to have little effect on the fatal dosage in animals, in severe asphyxia the toxicity of the drug was greatly increased. The lack of a beneficial effect from epinephrine suggests that clinically asphyxia or cyanosis should be treated first by oxygen inhalation before giving quinine intravenously.

Shock or hemorrhage, occurring as a result of injury or operation may accompany malaria, and both of these conditions increased the sensitivity of animals to quinine. In these conditions, epinephrine added to the quinine was effective in rabbits, but not so effective in cats.

CONCLUSIONS

1. The sensitivity of cats and rabbits to quinine was found to be greatly increased in non-hemorrhagic shock, after hemorrhage, in severe asphyxia, and in hyperthermia. In moderate asphyxia, the fatal dosage of quinine was not significantly different from that in normal animals.

2. Epinephrine given with quinine at the rate of 0.004 mgm. for each 2 mgm. of quinine was effective in increasing the fatal dosage of quinine in rabbits subjected to marked hemorrhage, non-hemorrhagic shock, and hyperthermia, but not to asphyxia. In cats, epinephrine was effective against quinine in hemorrhage and hyperthermia.

3. Of the 3 antagonists compared in rabbits, namely, epinephrine, neosynephrine, and calcium chloride, epinephrine was the only one which consistently mitigated the blood pressure lowering effect of quinine given intravenously in doses of 10 mgm./kgm. in hemorrhagic shock, non-hemorrhagic shock, hyper-

ANTAGONISTS FOR FATAL AND NON-FATAL DOSES OF QUININE INTRAVENOUSLY IN DEPRESSED CIRCULATORY STATES AND IN HYPERTHERMIA[1]

ROBERT H. DREISBACH

Department of Pharmacology and Therapeutics, Stanford University School of Medicine, San Francisco 15, California

Received for publication November 12, 1948

Although new anti-malarial agents are coming into widespread use, quinine will probably continue to be used, especially when intravenous use is indicated in severe falciparum malaria with coma or other cerebral symptoms (1). However, intravenous injection of the alkaloid might be hazardous, especially in pre-existing circulatory depression. Since this matter has not been previously explored, it was decided to obtain evidence on the toxicity of the alkaloid, and on the value of certain antagonists for its circulatory depression, in hemorrhage, asphyxia, hyperthermia, and shock. This paper presents the results obtained.

METHODS. The same general methods were followed as in a previous report (2). In addition to comparisons of the depressor effect of single doses (10 mgm./kgm.) of quinine in 0.9 per cent sodium chloride solution injected in 2 minutes, and the same dose of quinine mixed with an antagonistic agent, it was thought desirable that the most promising agent, epinephrine, should also be studied for possible antagonism to the fatal dose of quinine. For this purpose, the fatal dose of quinine hydrochloride was determined by continuous injection of a 1 per cent solution in normal saline solution at the rate of 2 mgm./kgm. per minute. This was then compared with the fatal dose of quinine when mixed with epinephrine in the ratio of 0.004 mgm. to 2 mgm. of quinine.

Both rabbits and cats were used. Anesthesia was produced with pentobarbital sodium, 35 mgm./kgm. being used intraperitoneally in cats, and intravenously, in rabbits. The various abnormal states were produced as follows: hemorrhagic shock by bleeding animals from the right femoral artery (blood pressures: 50–60 mm. Hg, rabbits and 70–80 mm. Hg, cats); moderate asphyxia with cyanosis and no change in blood pressure by allowing the animal to breathe through a 4-foot length of ½ inch rubber tubing, or by clamping partially the trachea; severe asphyxia with variable though moderate blood pressure changes by using a 6-foot tube; shock with hemorrhage by opening the abdomen and gently manipulating and tugging on the mesenteries and viscera (blood pressures: 50–60 mm. Hg, rabbits and 90–100 mm. Hg, cats). For hyperthermia the body temperature of the anesthetized animals was raised to 41°–42°C. by tying the animal supine on an electrically heated pad. Although small numbers of animals were used in each state, due to non-availability, differences in effects were significant in most cases.

Small Doses of Quinine and Antagonists in Various Abnormal States (table 1). Epinephrine (0.015 mgm./kgm.) was the only drug which consistently antagonized the blood pressure lowering effect of quinine (10 mgm./kgm.) and increased recovery of blood pressure in the various abnormal states studied. After hemorrhage in rabbits, epinephrine decreased the blood pressure fall from a control of

[1] Supported, in part, by a grant from the Therapeutic Research Committee of the Council on Pharmacy and Chemistry of the American Medical Association.

49 per cent for quinine alone, to 16 per cent. In non-hemorrhagic shock in rabbits, quinine produced a fall of blood pressure of 39 per cent, but with epinephrine added the fall was only 14 per cent. In partial asphyxia in rabbits, addition of epinephrine changed the blood pressure fall of quinine from 33 per cent to 15 per cent, and in hyperthermia, epinephrine decreased the quinine fall from 52 per cent to 22 per cent. Recovery of blood pressure was benefitted by

TABLE 1

Small doses of quinine and quinine with antagonists in various abnormal states

DRUGS*	RABBITS				CATS			
	No.	Trials	Aver. fall of blood pressure	Aver. recovery‡ of blood pressure	No.	Trials	Aver. fall of blood pressure	Aver. recovery‡ of blood pressure
Hemorrhage								
			per cent	*per cent*			*per cent*	*per cent*
Quinine	8	29	49 (9–73)†	86	3	11	26 (12–41)†	86
Quinine plus epinephrine	3	16	16 (0–29)	100	3	11	25 (0–53)	100
Non-hemorrhagic shock								
Quinine	9	48	39 (8–69)	89	2	9	17 (12–27)	91
Quinine plus epinephrine	4	27	14 (0–60)	100	2	9	8 (0–30)	95
Partial asphyxia								
Quinine	13	38	33 (4–69)	88	3	15	27 (15–49)	89
Quinine plus epinephrine	6	17	15 (0–37)	99	3	15	18 (0–33)	95
Hyperthermia								
Quinine	8	23	52 (21–83)	69	2	6	28 (21–35)	83
Quinine plus epinephrine	2	7	22 (6–38)	96	2	6	17 (6–28)	98

* Drugs (quinine 10 mgm./kgm., epinephrine 0.015 mgm./kgm.) were injected intravenously in 2 minutes.

† Range in parentheses.

‡ Compared with pre-injection level in mm. Hg.

epinephrine in each case. In cats, epinephrine was somewhat effective as an antagonist to quinine in non-hemorrhagic shock, asphyxia, and hyperthermia, but not after hemorrhage.

Neosynephrine (0.05 mgm./kgm.) and calcium chloride (10 mgm./kgm.) were also tried as antagonists to the blood pressure lowering effect of quinine, but were not significantly effective.

Fatal Doses of Quinine and Epinephrine in Normal and Abnormal States (table

2). In normal anesthetized rabbits and cats, the median fatal doses were 208 mgm./kgm. and 88 mgm./kgm., respectively. Addition of epinephrine raised the median fatal dosage of quinine in rabbits to 292 mgm./kgm.; in cats, to 128

TABLE 2

Fatal dosage of quinine and epinephrine in normal and abnormal states

DRUG	RABBITS			CATS		
	Range*	Median	Change (+ increase) (− decrease)	Range*	Median	Change (+ increase) (− decrease)
Controls						
	mgm./kgm.	mgm./kgm.	per cent	mgm./kgm.	mgm./kgm.	per cent
Quinine	191–218	208		80–102	88	
Quinine plus epinephrine†	288–334	292	+40	128–140	128	+45
Hemorrhage						
Quinine	67–110	76		64–86	69	
Quinine plus epinephrine†	118–178	146	+92	46–128	119	+72
Quinine after human plasma	55–230	128	+68			
Quinine plus epinephrine† after human plasma	66–133	104	+37			
Quinine after 6% acacia	67–234	100	+32			
Moderate asphyxia						
Quinine	150–206	188		94–116	102	
Quinine plus epinephrine†	104–136	120	−36	88–128	92	−10
Severe asphyxia						
Quinine	7–72	23		33–58	38	
Quinine plus epinephrine†	32–72	62	+170	50–70	66	+74
Non-hemorrhagic shock						
Quinine	72–180	90		22–110	71	
Quinine plus epinephrine†	202–212	208	+131	80–136	99	+39
Hyperthermia						
Quinine	28–52	50		52–94	56	
Quinine plus epinephrine†	114–166	150	+200	124–200	130	+132

* Three rabbits or 3 cats were used for each dosage range.

† Epinephrine, 0.004 mgm. for each 2.0 mgm. of quinine hydrochloride was injected intravenously per kgm. of body weight per minute.

mgm./kgm. After hemorrhage in rabbits, the median fatal dose for quinine alone was 76 mgm./kgm. and this was effectively raised to 146 mgm./kgm. by the addition of epinephrine. Epinephrine, however, was not so effective after

hemorrhage in cats. In moderate or severe asphyxia in either cats or rabbits, epinephrine was not effective as an antagonistic agent. In non-hemorrhagic shock the median fatal dose for quinine alone, in rabbits, was 90 mgm./kgm.; in cats, 71 mgm./kgm. After epinephrine was added to the quinine, the median fatal dose was 208 mgm./kgm. in rabbits, 99 mgm./kgm. in cats. In hyperthermia, the median fatal dose for quinine was 50 mgm./kgm. in rabbits, and 56 mgm./kgm. in cats. Addition of epinephrine raised the median fatal dose of quinine to 150 mgm./kgm. in rabbits, 130 mgm./kgm. in cats.

Discussion. In the various abnormal states studied, namely, non-hemorrhagic shock, hemorrhage, asphyxia, and hyperthermia, the toxicity and depressor effect of quinine were almost uniformly increased. Any of these conditions might be found in clinical malaria, either as a result of the disease process itself, or of injury, or other diseases. High fever, of course, is almost always present in severe malaria, and this was found to increase the toxicity of quinine. Epinephrine, however, was quite effective in raising the fatal dose, and in antagonizing the depressor effect of quinine, thus suggesting its use in clinical malaria with high fever when quinine must be given intravenously, and assuming a similar antagonism.

Partial asphyxia in malaria might easily result from the decreased oxygen carrying capacity of the blood due to the clumping or sludging of red blood cells, or to concomitant pneumonia, or other disease process. While moderate asphyxia seemed to have little effect on the fatal dosage in animals, in severe asphyxia the toxicity of the drug was greatly increased. The lack of a beneficial effect from epinephrine suggests that clinically asphyxia or cyanosis should be treated first by oxygen inhalation before giving quinine intravenously.

Shock or hemorrhage, occurring as a result of injury or operation may accompany malaria, and both of these conditions increased the sensitivity of animals to quinine. In these conditions, epinephrine added to the quinine was effective in rabbits, but not so effective in cats.

CONCLUSIONS

1. The sensitivity of cats and rabbits to quinine was found to be greatly increased in non-hemorrhagic shock, after hemorrhage, in severe asphyxia, and in hyperthermia. In moderate asphyxia, the fatal dosage of quinine was not significantly different from that in normal animals.

2. Epinephrine given with quinine at the rate of 0.004 mgm. for each 2 mgm. of quinine was effective in increasing the fatal dosage of quinine in rabbits subjected to marked hemorrhage, non-hemorrhagic shock, and hyperthermia, but not to asphyxia. In cats, epinephrine was effective against quinine in hemorrhage and hyperthermia.

3. Of the 3 antagonists compared in rabbits, namely, epinephrine, neosynephrine, and calcium chloride, epinephrine was the only one which consistently mitigated the blood pressure lowering effect of quinine given intravenously in doses of 10 mgm./kgm. in hemorrhagic shock, non-hemorrhagic shock, hyper-

thermia, and asphyxia, in agreement with the comparative antagonistic efficiency of these drugs for the depressor effect of quinine in normal rabbits.

4. Assuming similar reactions in man, these experimental results in depressed circulatory states, and possibly in hyperthermia, suggest cautious use of quinine intravenously in treating malarial patients with similar conditions, and the concurrent or prompt use of epinephrine intravenously as an antagonist, except in asphyxia.

REFERENCES

1. McGinn, S., and Carmody, J. T. B.: U. S. Nav. Med. Bull., 43: 1157, 1944.
2. Dreisbach, R. H., and Hanzlik, P. J.: This Journal, 83: 167, 1945.

COMPARATIVE CENTRAL DEPRESSANT ACTIONS OF SOME 5-PHENYL-5-ALKENYL BARBITURIC ACIDS

GORDON A. ALLES AND CHARLES H. ELLIS

Division of Pharmacology and Experimental Therapeutics, University of California Medical School, San Francisco, and the Laboratories of Gordon A. Alles, Ph.D., Pasadena

Received for publication November 13, 1948

The maximum anticonvulsant activity within the series of 5-phenyl-5-alkyl barbituric acids was found to be exhibited by the butyl derivative in the study of Alles, Ellis, Feigen and Redemann (1). This phenyl butyl barbituric acid exerted markedly less hypnotic action than related derivatives having more moderate anticonvulsive effectiveness. However, it now appears that the low solubility in water of the free phenyl butyl barbituric acid makes oral administration of this compound, even in the form of its sodium salt, only partially effective as compared with its effectiveness when injected intraperitoneally as a solution of the sodium salt. After oral administration of the sodium salt into the stomach a considerable amount of the salt is converted into the relatively insoluble free acid from reaction with the resting hydrochloric acid of the stomach or neutral buffering systems of the tissues of the gastrointestinal tract.

To search for compounds that might have greater water solubility, yet share with phenyl butyl barbituric acid its relatively high anticonvulsive effectiveness, study was extended to the phenyl unsaturated-alkyl barbituric acids. To value relative activities, pairs of corresponding saturated and unsaturated phenyl aliphatic barbituric acids were chosen for this study. The saturated alkyl compounds were selected from among those described in the previous study and the allyl compound was obtained from the commercially available hypnotic product. The two other unsaturated compounds were synthesized by Dr. C. Ernst Redemann and Dr. Roland N. Icke in Pasadena, by condensing the properly disubstituted malonic ethyl esters with urea in a sodium methylate solution, then purifying the products formed. Identity and purity of the compounds used were established by comparing melting point observations with the literature data and by analyses as shown in table 1.

Anticonvulsant Activities. The evaluation of this type of central depressant action of the compounds was carried out by methods previously described in detail by Alles, Ellis, Feigen and Redemann (1, 2). It involved measuring the change produced by the compound on the duration of flow of a 50 milliampere current required to cause a tonic convulsion in rabbits. The current was applied through two temporal electrodes and was supplied by an Offner electroshock therapy apparatus modified to provide a suitable range of shock durations. The compounds were injected intraperitoneally in the form of aqueous solutions of the respective sodium salts, and the re-evaluation of the convulsive threshold was begun one hour after injection. The control threshold was established for each animal as shown in table 2. Each rabbit was tested with each of the group

TABLE 1

Identification and properties of derivatives

NH—CO—NH CO—C—CO R_1 R_2	MELTING POINT °C		ANALYSIS						SOLUBILITY IN H_2O AT 37°C
			% Carbon		% Hydrogen		% Nitrogen		
	Found	Reported	Found	Calcd	Found	Calcd	Found	Calcd	*mgm /100 cc*
Phenyl propyl	193–4	193–4[a]	—	—	—	—	11 29	11 36	32
Phenyl allyl	157–8	154–5[b]	—	—	—	—	—	—	278
Phenyl butyl	213–4	213–4[a]	64 52	64 52	6 57	6 25	10 83	10 77	12–13
Phenyl crotyl	208–9	—	65 30	65 10	5 58	5 46	10 70	10 82	32–34
Phenyl *iso*butyl	175–6	175–6[a]	64 60	64 52	6 10	6 25	10 73	10 77	30
Phenyl β methallyl	199–200	203–5[c]	65 45	65 10	5 46	5 46	—	—	14–18

[a] Alles, Ellis, Feigen and Redemann This Journal, 89 356, (1947)
[b] Horlein and Krop U S #1,056,793, Mar 25, 1913
[c] Doran and Shonle, J Am Chem Soc, 59 1625, (1937)

TABLE 2

Comparative anticonvulsant activities of phenyl aliphatic barbituric acids on threshold for electrically induced convulsions in rabbits

COMPOUND NH—CO—NH CO—C—CO Ph R	DOSE	DURATION IN SECONDS OF 50 MILLIAMPERE CURRENT TO CAUSE TONIC CONVULSION							
			Animal number						Mean
			2 0	2 1	2 2	2 3	2-4	2 5	
	mM /kgm								
Propyl	0 1	Initial	0 2	0 1	0 1	0 05	0 2	0 4	0 17
		Final	3 7	0 9	0 4	0 2	3 7	3 7	2 10
Allyl	0 1	Initial	0 2	0 2	0 1	0 4	0 2	0 2	0 22
		Final	1 8	1 8	1 8*	1 8*	3 7	30 0*	6 81
Butyl	0 1	Initial	0 4	0 4	0 2	0 1	0 2	0 4	0 25
		Final	7 5	3 7	0 4	3 7	30 0	3 7	8 17
	0 2	Initial	0 2	—	—	—	—	0 1	0 15
		Final	30 0	—	—	—	—	30 0	30 00
Crotyl	0 1	Initial	0 2	0 1	0 2	0 2	0 4	0 2	0 22
		Final	30 0	0 4	1 8	3 7	3 7	3 7	7 22
	0 2	Initial	0 2	—	—	—	—	0 2	0 20
		Final	30 0	—	—	—	—	30 0	30 00
*iso*Butyl	0 1	Initial	0 2	0 4	0 2	0 1	0 2	0 4	0 25
		Final	3 7	0 4	0 9	0 9	0 4	0 4	1 12
β Methallyl	0 1	Initial	0 2	0 1	0 1	0 2	0 4	0 2	0 20
		Final	3 7	0 4	0 4	0 9	1 8	3 7	1 82
Control run	—	Initial	0 2	0 2	0 1	0 2	0 2	0 2	0 19
		Final	0 2	0 4	0 2	0 4	0 2	0 2	0 27

* Showed marked drowsiness or deeper hypnotic effects

of compounds on different days with at least one week between determinations on the same animal. The initial threshold was redetermined each time just before administration of a compound. In view of the previous investigation of Alles, Ellis, Feigen and Redemann (1), which showed that the saturated phenyl alkyl barbituric acids included in the present study exhibit definite anticonvulsive activity at 0.1 millimols per kilogram of body weight, this dosage was chosen for the comparison of these compounds with their corresponding unsaturated homologs.

It can be seen from table 2, that the introduction of unsaturation into the alkyl group by use of the corresponding alkenyl derivative does not greatly alter the anticonvulsant activity from that of the saturated alkyl derivative. In accord with this, it is to be particularly noted that comparison of phenyl crotyl

TABLE 3

Duration of actions on convulsant thresholds in rabbits of phenyl butyl and phenyl crotyl barbituric acids after intraperitoneal injections of 0.1 millimols/kilogram

COMPOUND	INTERVAL BETWEEN DOSE AND START OF THRESHOLD SHOCKS		DURATION IN SECONDS OF 50 MILLIAMPERE CURRENT TO CAUSE TONIC CONVULSION					
			Animal Number					Mean
			2-0	2-1	2-3	2-4	2-5	
	hours							
Phenyl butyl	1	Initial	0.4	0.4	0.1	0.2	0.1	0.24
		Final	7.5	3.7	3.7	30.0	3.7	9.72
	4	Initial	0.2	0.1	0.05	0.1	0.2	0.13
		Final	1.8	0.4	0.05	0.4	0.9	0.71
Phenyl crotyl	1	Initial	0.2	0.1	0.2	0.4	0.2	0.22
		Final	30.0	0.4	3.7	3.7	3.7	8.30
	4	Initial	0.2	0.4	0.05	0.4	0.1	0.23
		Final	0.9	0.4	0.05	0.9	0.9	0.63

barbituric acid with phenyl butyl barbituric acid at the dose of 0.2 millimols per kilogram showed the protection against the 50 milliampere current to be complete (over 30 seconds) in both instances.

Because of an apparent shorter duration of hypnotic actions of the alkenyl compounds when compared with corresponding alkyl homologs, an attempt was made to find how anticonvulsant activities compared when a period longer than one hour elapsed before re-evaluating the convulsive threshold. This comparison was carried out with only one pair of compounds, namely, phenyl butyl and phenyl crotyl barbituric acids. Re-evaluation of the convulsive thresholds was made one hour and four hours after injecting the compounds. The data are given in table 3, and it may be noted that after four hours the elevation over the control threshold is no longer marked, and that the reduction in the convulsive threshold at four hours from that at the one hour level is almost complete and is similar for both compounds.

Hypnotic Activities and Acute Toxicities. Relative central depressant action of the compounds was also investigated in animals by determining hypnotic effects in mice. Doses given were further increased to value acute toxicity. Hypnosis was considered to be present if the animal was unable to right itself within 60 seconds after being placed on its back. Solutions of the sodium salts in water were injected intraperitoneally into white mice kept in an environmental temperature of 29–30°C. during the period of hypnosis and, routinely, for 24 hours after the drug injection. Experience has indicated that data obtained at different times and on different series of animals is not entirely comparable, so the saturated compounds previously reported were reinvestigated as if no earlier work had been done. Thus, the entire series of compounds included in this study were examined for hypnotic activity and acute toxicity over a period of less than two months on mice of the same strain from the same source. Calculation of HD-50 and LD-50 has made use of the method of Bliss (3).

TABLE 4

Comparative hypnotic and lethal effects of some 5-alkyl- and 5-alkenyl-5-phenyl barbitals

COMPOUND	INTRAPERITONEAL DOSAGE IN MILLIMOLS/KGM.								HD-50 ±S.E.	LD-50 ±S.E.
	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6		
-Propyl	0/0*	5/0	9/0	10/0	10/1	10/6	10/9	10/10	.406 ± .044	1.170 ± .039
-Allyl	0/0	5/0	7/0	10/1	10/1	10/3	10/7	10/9	.429 ± .047	1.256 ± .069
-Butyl	0/0	4/0	10/0	10/1	10/4	10/6	10/7	10/10	.417 ± .035	1.111 ± .060
-Crotyl	0/0	1/0	9/0	9/1	9/1	10/3	10/6	10/5	.514 ± .054	1.482 ± .159
-*iso*Butyl	0/0	8/0	10/0	10/4	10/4	10/10	10/10	10/10	.340 ± .030	.918 ± .048
-β-Methylallyl	0/0	4/0	10/0	10/1	10/3	10/2	10/9	10/8	.417 ± .035	1.217 ± .069

* The numbers represent those among 10 mice tested which lost righting reflexes/which died.

Examination of table 4 shows that the hypnotic activity, and to a greater extent, the lethal activity, is decreased by the presence of an unsaturation of the aliphatic group. When compared to the propyl, butyl and *iso*butyl compounds, the corresponding allyl, crotyl and β-methallyl unsaturated compounds show hypnotic activities of 95, 81 and 82 per cent, and lethal activities of 93, 75 and 76 per cent. This difference in activity is not great, but analysis of variance shows the overall trend to be fairly significant with hypnotic P equal 0.10 and lethal P equal 0.02.

Central Depressant Activities in Man. Only those compounds which showed in rabbits the most pronounced anticonvulsant activity with a minimum of hypnotic activity were examined for some measurable central depressant effects in man. The compounds used were phenyl butyl and phenyl crotyl barbituric acids for, although phenyl allyl barbituric acid showed similar anticonvulsant activity in rabbits, this activity was accompanied by a marked degree of hypnotic activity as well.

Following the ideas of Simonsen and Enzer (4, 5), finger tapping rate and critical fusion frequency of flicker were used as measures related to the functioning

of motor centers and of visual centers, respectively. The finger tapping rate was measured with a telegraph key connected to an impulse counter in circuit with a one minute fixed-interval automatic reset timer switch. The key was stiffened to eliminate tremor or trilling effects. The critical fusion frequency was measured with an electronic apparatus of the design of Henry (6) as modified by Keighley and Clark (7) which permits controlled variation of frequency of flicker of a neon bulb light held at constant intensity. This apparatus permits

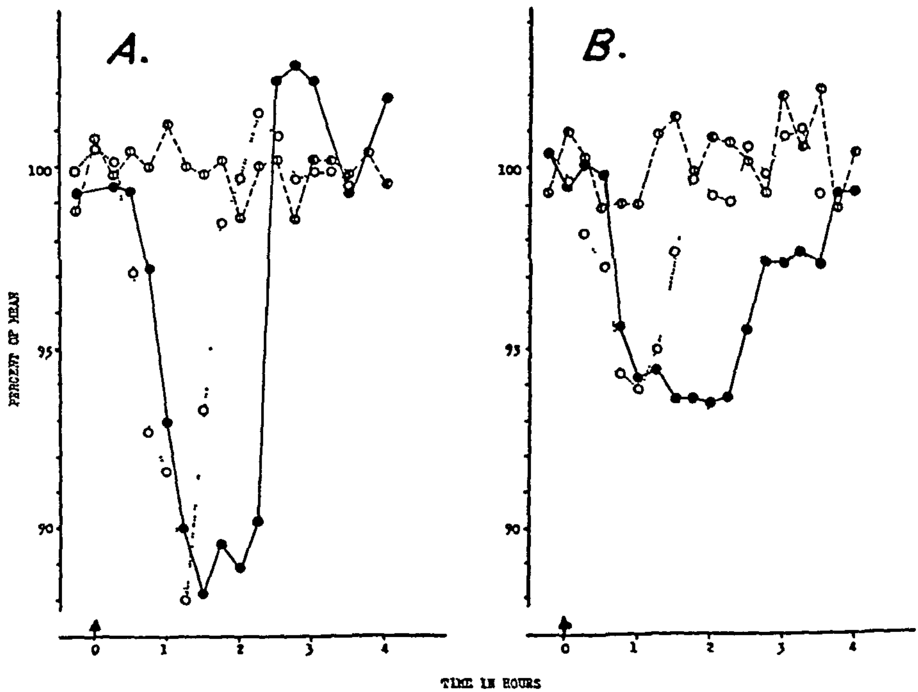


FIG. 1. The curves indicate the influence of oral administration of the sodium salts of 5-phenyl-5-butyl barbituric acid (solid lines) and 5-phenyl-5-crotyl barbituric acid (dotted lines) on: A. frequency of tapping, and B. critical fusion frequency of flicker. Each point represents the mean of three readings, and is expressed as the percentage of the expected mean value by comparison with the regression curve defined by the means of the first two and last two determinations. Time is in hours after the oral administration of 350 mgm of the barbital as the sodium salt in water solution. For comparison, control run observations without medication (broken lines) are included.

accurate frequency calibration by comparison with standard 60 cycle alternating current.

Direct comparison of the effects in the same person (figure 1) shows that the depression of both tapping frequency and critical fusion frequency which results from an orally administered 350 mgm. dose of phenyl crotyl barbituric acid in the form of its sodium salt, occurs earlier than the depression which results from a similar dose of the sodium salt of phenyl butyl barbituric acid. As shown in the figure, the duration of depression produced by phenyl crotyl barbituric acid

is shorter. Subjectively, the feeling of dizziness and lack of attention produced by these compounds is similar, and the maximum of these subjective effects coincides with the maximum depression in tapping rate and critical fusion frequency.

The systemic activity of orally administered barbituric acids on their salts must be related to their absorption from the gastrointestinal tract, and this would appear to be greatly influenced by the relative ease of absorption of the

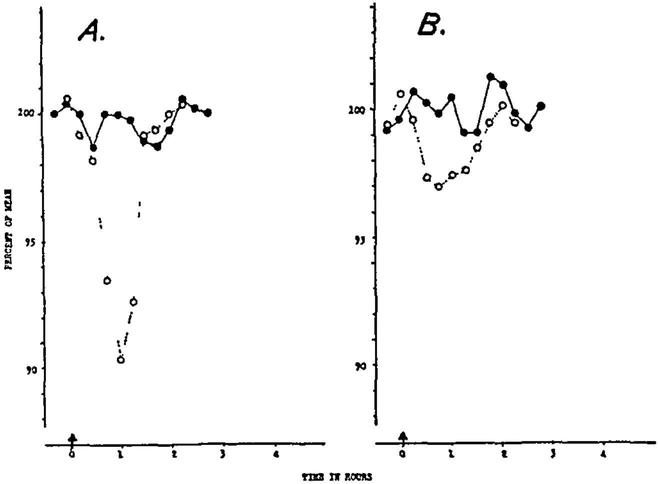


FIG 2 The curves indicate the influence of oral administration of the free acids of 5-phenyl 5 butyl barbituric acid (solid lines) and 5-phenyl-5 crotyl barbituric acid (dotted lines) on A frequency of tapping, and B. critical fusion frequency of flicker. Same subject as in fig 1, and administration of 350 mgm of the barbital as the finely divided free acid in water suspension

free acids and the extent to which the compounds are present in this form. Comparison of the depressant effects of phenyl butyl and phenyl crotyl barbituric acids when administered in the form of the free acids with the effects which result from administration of the respective sodium salts shows the phenyl crotyl compound to be much superior to that of the phenyl butyl compound. It can be seen from figure 2 that phenyl crotyl barbituric acid, when administered as free acid, was only somewhat less effective than when administered as a solution of its sodium salt Administration of phenyl butyl barbituric acid in the form of free acid, however, resulted in only a negligible degree of depressant action

Anderson, Chen and Leake (8) reported that certain barbiturates produce a

reduction in basal metabolic rate. The butyl and crotyl derivatives of interest to this study have been compared in this respect following oral administration in the form of the sodium salts, and both showed a moderate reduction in the rate of oxygen consumption. There were no significant changes in blood pressure, pulse rate, or body temperature during this period. Phenyl butyl barbituric acid caused a reduction of about 10 per cent in respiratory minute volume after 90 minutes, while phenyl crotyl barbituric acid caused a reduction of about 16 per cent after 60 minutes. Whereas both compounds reduced tidal air volume to some extent, the respiratory frequency was unchanged after the butyl com-

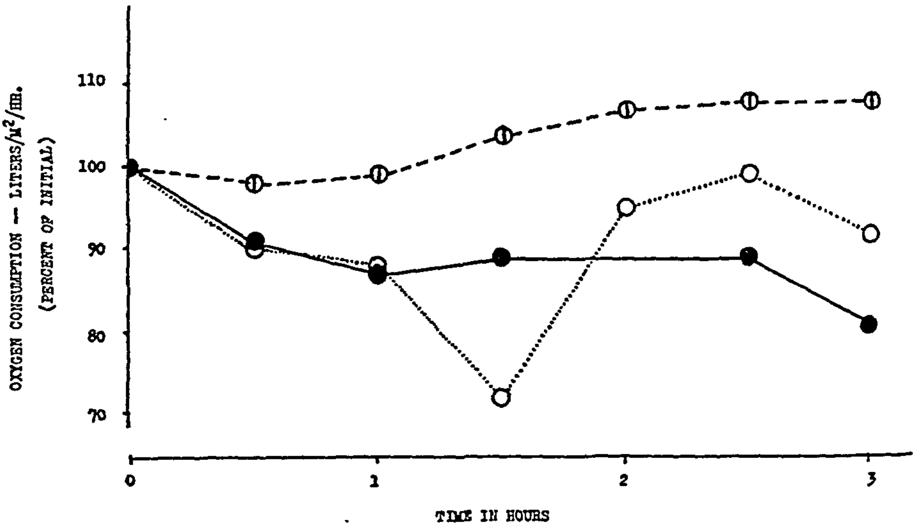


FIG. 3. The curves indicate the influence of oral administration of the sodium salts o 5-phenyl-5-butyl barbituric acid (solid line) and 5-phenyl-5-crotyl barbituric acid (dotted line) on oxygen consumption. Oxygen consumption is expressed as the percentage of the initial determination. Time is in hours from the administration of 350 mgm. of the barbital as the sodium salt in water solution. For comparison, control run observations without medication are included.

pound, but increased about 20 per cent following administration of the crotyl compound.

DISCUSSION. A series of compounds in which the alkyl group of one of the 5-phenyl-5-alkyl barbituric acids is replaced by a corresponding alkenyl group, was studied. In the case of the allyl and crotyl derivatives this replacement resulted in a considerable increase in solubility in water of the free barbituric acids, which should be of importance with regard to the effective absorption of the compound following oral administration.

It is of interest that, while the hypnotic and toxic activities of the phenyl allyl and phenyl crotyl barbituric acids are less than those of the corresponding alkyl compounds, the anticonvulsive activities are not markedly different between corresponding phenyl alkyl and phenyl alkenyl compounds. The range, therefore, between desirable anticonvulsant effectiveness and undesirable hypnotic activity of such compounds for use as anticonvulsive agents is increased by the

substitution of the alkenyl group, particularly in the case of phenyl crotyl barbituric acid While this range has been increased also in the case of phenyl allyl barbituric acid, the compound is still hypnotic in the rabbit with the doses required to demonstrate anticonvulsive effectiveness

The finding of diminished hypnotic action with alkenyl compounds is in accord with the observations of Shonle and Waldo (9) within a series of 5 ethyl 5 alkenyl barbituric acids Their study was restricted to compounds containing unsaturated secondary pentyl or hexyl groups, which may serve to account for their finding of convulsive properties among the unsaturated compounds studied In our studies no convulsions were noted to result from any of the phenyl alkenyl compounds used in the dosages which were tried

As compared with the effects of phenyl butyl barbituric acid, the earlier onset and shorter duration of the depressant action that is produced by phenyl crotyl barbituric acid on both motor and sensory cortex functions may be related to the greater solubility of the phenyl crotyl compound, apparently resulting in earlier absorption of an effective amount of the compound, and earlier detoxification or excretion that will tend to shorten the duration of action That the unsaturated compounds exhibit a shorter duration of action is seen not only in the depression of motor and sensory cortical function in man, but in the reduction of metabolic rate in man, and in the hypnotic response in mice The differences are not very great, and can possibly be accounted for by an increased facility for detoxification or excretion which may also be related to the greater water solubility of the unsaturated compounds

SUMMARY

1 Additional evidence, from studies of the comparative central actions of phenyl alkenyl barbituric acids, is presented to show that the mechanism which raises the convulsant threshold is different from the mechanism producing hypnosis

2 Anticonvulsant activity of the phenyl alkenyl barbituric acids appears to be maximal with the four membered straight alkyl chain compound, 5 phenyl-5 crotyl barbituric acid

3 The phenyl crotyl compound is more soluble in water than the corresponding phenyl butyl compound, and is more effectively absorbed after oral administration, when both are given in the form of their free acids

REFERENCES

1 Alles, G A, Ellis, C H, Feigen, G A, and Redemann, M A This Journal, 89. 356, 1947
2 Alles, G A, Ellis, C H, Feigen, G A, and Redemann, M A Univ Calif Publ Pharmacol, 2 173, 1946
3 Bliss, C I Quart J Pharm & Pharmacol, 11 192, 1938
4 Simonsen, E, and Enzer, N Arch Int Med, 68 498, 1941
5 Simonsen, E, and Enzer, N J Ind Hyg and Toxicol, 23 83, 1941
6 Henry, F J Exper Psychol, 31 538, 1942
7 Keighley, G, and Clark, W G Fed Proc, 5 54, 1946
8 Anderson, H H, Chen, M Y, and Leake, C D This Journal 40 215, 1930
9 Shonle, H A, and Waldo, J H J Am Chem Soc, 55 4649, 1933

REDUCTION AND OXIDATION OF CHLORAL HYDRATE BY ISOLATED TISSUES *IN VITRO*

THOMAS C. BUTLER

Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University, Baltimore, Maryland

Received for publication November 15, 1948

In an earlier report (1) it was shown that chloral hydrate undergoes rapid transformations in the dog, a small part being oxidized to trichloroacetic acid and the greater part being reduced to trichloroethanol. Evidence was presented indicating that the trichloroethanol so produced is responsible, in large part at least, for the characteristic depressant effects of chloral hydrate. The object of the present investigation was to find the sites at which chloral hydrate undergoes these chemical reactions, especially the pharmacologically important reaction of reduction. The method of study consisted in measurement of trichloroethanol and trichloroacetic acid produced from chloral hydrate by isolated tissue preparations *in vitro*. The study included tissues of the dog and of the rat, investigation of the latter species being the more extensive.

METHODS. The animals were allowed free access to food up to the time when they were killed for the removal of tissue samples. Liver, kidney, brain, and spleen were used in the form of thin slices cut with a razor blade. Whole diaphragms of rats were cut into several pieces before use. In the preparation of rat testis the tubules were teased out in the suspending medium.

To each gram of the solid tissues was added approximately 4 cc. of a suspending medium having the following composition: sodium chloride, 0.125 M; potassium chloride, 0.005 M; calcium chloride, 0.003 M; magnesium sulfate, 0.001 M; sodium phosphate buffer, pH 7.4, 0.01 M; glucose, 0.01 M; chloral hydrate, 0.002 M. In the study of blood, coagulation was prevented by the addition of 5 mgm. of potassium oxalate or 0.1 mgm. of heparin per cc. of blood. To 9 volumes of whole blood or plasma was added 1 volume of a solution having the same composition as that above except that the concentration of chloral hydrate was 0.02 M. After the addition of the medium containing chloral hydrate, all of the tissues were shaken in flasks for 30 min. at 38°C. The gas phase was oxygen.

At the end of the period of shaking, the contents of the flasks were centrifuged and analyses for trichloroethanol and trichloroacetic acid performed on the supernatant liquid. In the case of whole blood the analyses were performed on the separated plasma. The chemical methods previously described (1) were used, sometimes with minor modifications that permitted the use of smaller samples or the attainment of greater sensitivity. Where values of zero are entered in table 1 for trichloroethanol or trichloroacetic acid, the concentrations could not have exceeded 3×10^{-5} moles per l.

RESULTS AND DISCUSSION. As shown in table 1, all of the tissues studied, both of the dog and of the rat, are capable, under aerobic conditions, of reducing chloral hydrate to trichloroethanol. The reaction even occurs in the blood cells.[1]

[1] In the previously reported work on the metabolic fate of chloral hydrate (1), the only precaution taken against the conversion of chloral hydrate to trichloroethanol in the blood samples *in vitro* was to centrifuge the blood and separate the plasma within a period not

The table indicates considerable differences in the rates at which the various tissues produce trichloroethanol However, on account of the abnormal conditions and the different degrees of cellular injury in the preparations, it is probably not justifiable from these data to make any quantitative estimates as to the

TABLE 1

Concentrations of trichloroethanol and trichloroacetic acid appearing in the suspending media or plasma after 30 min at 38°C

The initial concentration of chloral hydrate in the suspending media and in blood was 200×10^{-5} moles per l

TISSUE	ANIMAL	CONCENTRATION (MOLES $\times 10^{-5}$ PER L)	
		Trichloroethanol	Trichloroacetic Acid
Liver	Rat 1	53	9
	Rat 2	68	9
Kidney	Rat 2	62	4
	Rat 3	66	4
Diaphragm	Rat 3	40	0
	Rat 4	36	0
Brain (entire)	Rat 2	12	0
	Rat 3	17	0
Testis	Rat 5	68	0
	Rat 6	61	0
Spleen	Rat 7	46	0
	Rat 8	63	0
Whole blood (heparin)	Rat 3	19	0
	Rat 7	25	0
Liver	Dog 1	47	4
Kidney cortex	Dog 1	52	6
Cerebral cortex	Dog 1	9	0
Whole blood (heparin)	Dog 1	12	0
	Dog 2	13	0
Whole blood (oxalate)	Dog 2	11	0
Plasma (heparin)	Dog 1	0	0
	Dog 2	0	0

proportion of administered chloral hydrate reduced in any particular organ in the intact animal Nevertheless, it does appear that no single organ plays an exclu-

exceeding half an hour The results of the following experiment make it appear unlikely that under the conditions of the previous work any significant errors could have arisen from changes taking place after collection of the blood samples A dog was given an intravenous dose of 0 8 millimoles (132 mgm) per kgm of chloral hydrate An oxalated blood sample taken after 6 min was divided into two parts The first was cooled for 5 min in an ice bath and centrifuged immediately thereafter The second was allowed to stand 30 min at room temperature without agitation before centrifugation Analyses of the plasmas gave the following results trichloroethanol, 59 mgm per l in the first and 56 mgm per l in the second, chloral hydrate, 152 mgm per l in the first and 142 mgm per l in the second These differences are within the limits of error of the methods and there is no indication that any further production of trichloroethanol occurred *in vitro*

sive, or even a predominant, role in carrying out the reaction. The widespread participation of tissues throughout the body is doubtless responsible for the rapidity with which the animal can reduce chloral hydrate.

Since trichloroethanol is produced by the brain cells, it might be expected that there would be a period after the administration of chloral hydrate when the concentration of trichloroethanol in these cells would not be proportional to that in the plasma. This is a factor that might be responsible in part for the observed discrepancies between the neurological condition of dogs and the plasma concentration of trichloroethanol soon after the administration of chloral hydrate (1).

It is of interest that the capacity of reducing chloral hydrate is not limited to animal cells. Reduction of chloral hydrate by fermenting yeast was demonstrated by Lintner and Lüers (2), concentrations of trichloroethanol as high as 2 gm. per l. being produced.

In contrast to the reduction reaction, the oxidation of chloral hydrate to trichloroacetic acid takes place only in certain specific sites. Of those tissues studied, only the liver and kidney of the dog and of the rat showed any production of trichloroacetic acid. It is of course conceivable that the reaction may also occur in tissues other than those investigated. Furthermore, it is conceivable that the sites at which chloral hydrate can be oxidized may not be the same in all species of animals.

SUMMARY

Measurements have been made of the trichloroethanol and trichloroacetic acid produced from chloral hydrate *in vitro* by isolated tissue preparations of the dog and of the rat.

All of the tissues studied were capable under aerobic conditions of reducing chloral hydrate to trichloroethanol.

Oxidation of chloral hydrate to trichloroacetic acid was demonstrated only in preparations of liver and kidney of both species.

REFERENCES

1. BUTLER, T. C.: THIS JOURNAL, 92: 49, 1948.
2. LINTNER, C. J., AND LÜERS, H.: *Ztschr. f. physiol. Chem.*, 88: 122, 1913.

POTENTIATION OF EFFECTS OF EPINEPHRINE BY FLAVONOID ("VITAMIN P"-LIKE) COMPOUNDS. RELATION OF STRUCTURE TO ACTIVITY[1,2]

WILLIAM G. CLARK AND T. A. GEISSMAN

Scripps Metabolic Clinic, La Jolla, Calif. and the Department of Chemistry, University of California at Los Angeles

Received for publication November 15, 1948

In 1936 Bacq (1, 2) showed that certain polyphenols containing *ortho* and *para* hydroxyl groups, such as pyrogallol, which may serve as epinephrine anti-oxidants *in vivo*, sensitize animals to and prolong the effects *in vivo* of epinephrine and sympathetic nerve stimulation. *Meta*-compounds such as resorcinol and phloroglucinol were inactive. Bacq interpreted the results as an inhibition of oxidation and prolongation of the action of epinephrine and sympathin at smooth muscle receptors.

Clark and Raventos (3) confirmed the finding of Bacq that pyrogallol prolongs the action *in vivo* of epinephrine and since then Herman, Vial and Chatonnet (4) showed that pyrogallol prolongs the blood pressure responses to epinephrine Several investigators have shown sensitization to and prolongation of responses *in vivo* to epinephrine by other epinephrine anti-oxidants such as ascorbic acid (5–9) and sodium bisulfite (10).

Since 1940, Lavollay *et al* and Parrot *et al* have proposed that the mechanism of action of "vitamin P (vitamin C_2)"-like compounds, most of which like pyrogallol also are polyhydric phenols, is by prolongation *in vivo* of the action of epinephrine, sympathin or one of their metabolic products such as adrenochrome, by retarding their inactivation. This in turn was said to cause decreased capillary fragility, capillary fluid filtration ("permeability") and bleeding time. In an extensive series of papers which have been reviewed in several places (11–16), they support this hypothesis by experiments designed to demonstrate the inhibition of the oxidative destruction *in vitro* and the potentiation *in vivo* of the effects of epinephrine by "vitamin P"-like compounds, particularly *d*-epicatechin.

Whether this interpretation of the biological action of the "vitamin P"-like substances is correct or not, remains to be demonstrated. That it may in part explain at least some of the pharmacological and physiological effects of flavonoids has been supported by the observations of Torres (17), Valdecasas and

[1] Published in preliminary form in Fed Proc , 7: 21, 1948 and in Nature, 163: 36, 1949

[2] Supported by grants from the California Fruit Growers Exchange, U S Public Health Service, the Roche Anniversary Foundation and S B Penick Co We are indebted to Prof T R. Seshadri, Waltair, S India, for generously supplying several of the compounds, to Profs J Lavollay, Paris and F G Valdecasas, Barcelona, for their many informative communications, to the Public Health and Welfare Section, Supreme Commander for the Allied Powers, and the Takeda Pharmaceutical Industries, Ltd , Japan, for compounds and Japanese translations

Muset (18–20), Wilson, Mortarotti and De Eds (21) and Fuhrman and Crismon (22). Opposed to this interpretation is the work of Merlini (23) and certain conflicting reports of Lavollay and Parrot themselves (14, 24–29), concerning whether or not flavone derivatives influence the classical effects of epinephrine in the intact animal, in contradiction to the chromane derivative, *d*-epicatechin. Certain substances which increase the vasoconstrictor phase of vasomotion of the capillary beds, such as epinephrine and adrenochrome, also are known to decrease capillary fragility and bleeding time (12–16, 30–34) and if flavonoid compounds can perform the function of sensitizing arterioles and pre-capillaries to or prolong the effects of epinephrine and sympathin, the mechanism of their biological action might be more clearly understood.

It has been impossible to state, on the basis of results so far published, whether the so-called "vitamin P" substances are dietary factors, or whether they act as specific drugs which modify the course of various pathological states which have been studied. One of the difficulties has been the lack of a reproducible assay for estimating "vitamin P deficiency" symptoms and their amelioration. "Capillary fragility" measurements are of questionable reliability and meaning, according to the recent critical review of Munro *et al.* (35).

An examination of the molecular structures of those substances which have been reported in the literature to be effective in decreasing capillary fragility, points to the possibility that there may be no common, single mechanism of action of these substances.

In order to isolate for detailed study one of the many effects claimed for the flavonoid-like substances, an examination has been made of some 70 compounds for their ability to increase the magnitude of response to and duration of the relaxation of the isolated mammalian intestinal segment by epinephrine. Lavollay (36), Muset and Valdecasas (18–20), Torres (17), Wilson, Mortarotti and De Eds (21) employed similar methods for studying epinephrine potentiation by flavonoid compounds but made no systematic study of molecular configuration. Lavollay, who has been the only prior investigator to consider the effects of variations in molecular structure, has drawn some conclusions concerning structure and activity, basing them upon studies of a limited and widely diversified group of compounds. His concept of structure and activity is treated in the discussion below. Lavollay (11) found a parallelism between the inhibition of autoxidation of epinephrine by flavonoids *in vitro* (manometric, colorimetric) and as measured by the prolongation of epinephrine response of isolated intestine. Hence we also included some spectrophotometric studies of the copper-catalyzed autoxidation of epinephrine *in vitro*.

METHODS. 1. *Intestinal Segment Assay of Potentiation of Epinephrine Action.* The use of the intestinal segment for measuring the response to epinephrine and other drugs is one of the most common preparations in the pharmacology laboratory but to our knowledge no one had described a strictly quantitative dose-response relationship for the isolated mammalian intestinal segment. Since a long time was required to develop our method it was thought worthwhile to describe the essential details.

Segments of rabbit ileum approximately 5 cm. long were removed from 12-hour fasted

rabbits sacrificed by the intravenous injection of eserine sulfate (2 mgm per kgm), which seemed to promote more rapid recovery of vigorous, constant rhythmic activity in the subsequently isolated segments The segments were mounted in smooth muscle chambers of 85 cc capacity, in Tyrode's solution at 38°C and gassed with 95 per cent O_2 + 5 per cent CO_2, which by its buffering action with the bicarbonate of the Tyrode's solution maintained the pH at 7 2 Under the experimental conditions described, the isolated segments gave vigorous, constant rhythmic contractions and drug responses for 6–14 hours

A stock of Tyrode's solution (40 to 60 l) was made up for each experiment, from the usual stock concentrates When freshly made up, the Tyrode's solution had a pH of 8 or more, necessitating gassing before use and during the experiment, in addition to vigorously gassing each smooth muscle chamber Four intestinal segments were mounted in individual chambers, immersed in a water bath at 37°C The Tyrode's solution passing from the reservoir to the chambers was pre warmed

Early in the work it was observed that the addition of copper ion to the Tyrode's solution eliminated a certain lack of reproducibility of the results, presumably because of the presence of small but inconstant amounts of copper in the distilled water, the salts used in the Tyrode's solution or the substances being tested Subsequently, it was found that 1 microgram of copper sulfate per cc of Tyrode's solution was the optimal amount This is of interest in the light of the observations of Friedenwald and Buschke (37), which were interpreted as showing that the effect of epinephrine on isolated intestine is conditioned by the presence of copper It is important to point out that in the absence of this added copper, some of the more active compounds prolong the epinephrine response so much that it is impossible to compare their activities

Kymograph records of the responses were made in the usual way, using ink writing pen points

The epinephrine was made up as the hydrochloride in distilled water from commercial ampules[3] or from pure powder[4] The amounts of epinephrine (5–10 micrograms) added were adjusted to give a recovery time of 0 5–2 0 minutes (in the absence of inhibitor) The test substances were added before the addition of epinephrine, as propylene glycol solutions containing 0 1–10 0 mgm /cc, in amounts ranging up to 10 mgm /85 cc chamber, depending upon activity, toxicity or solubility The amounts of propylene glycol used had no effect on the preparation or its response to epinephrine in the presence or absence of the test drugs Control responses to epinephrine were determined frequently throughout all experiments, until they were reproducible before the next test sample was added After each test, the chambers were washed out and after higher concentrations of test substances more frequent washings and waits were necessary before the epinephrine control responses returned to normal More consistent responses were obtained if tests were made as soon as possible after washing, presumably because of the epinephrine stabilizing effects of accumulated metabolites and mucosal slough (38)

In addition to flavonoid like compounds, a number of other known anti oxidants such as glutathione and ascorbic acid were tested A number of compounds known (37) to enhance and prolong the effect of epinephrine on isolated intestine also were included, such as 8 hydroxyquinoline and sodium diethyldithiocarbamate

The half recovery times from the relaxation due to epinephrine alone were compared with those when the test substances were added prior to, and in addition to epinephrine The number of times the half recovery times from epinephrine plus test drug exceeded those from epinephrine alone, in the case of all four segments, were averaged and plotted against the concentrations of the test drugs

Rutin (quercetin 3 rhamnoglucoside) was selected as the reference compound of unit activity because it was readily available in pure form in quantity, and because of its current

---

[3] Parke, Davis, 1 1000 Epinephrine hydrochloride

[4] Wilson Laboratories, Chicago

popularity arising from the rediscovery by Griffith, Couch and Lindauer (39) of Sevin's observation (40) that rutin exerts anti-capillary-fragility action. Several compounds were compared with rutin in each experiment and the average prolongation of the half-recovery times were plotted on rectangular coordinate or log-log graph paper. Each point on each curve of all such assays represented several determinations, since 4 segments were measured simultaneously for each compound, and in most cases represented the average of several such quadruplicate determinations, as illustrated in the protocol for Exp. 37, table I. Under these conditions, the averages gave straight lines diverging from zero on rectangular coordinate paper and parallel straight lines on log-log paper. Except for an occasional widely disparate value, the results for each substance were reproducible when repeated in different experiments, so far as the actual values in "rutin units" were concerned. It was found that the prolongation times for all compounds in all experiments could not be averaged and plotted on a master graph from which the activities could be measured, presumably because of differences in responses between individual animals. For this reason, the responses of the segments to rutin were compared with other test substances in each experiment, from which the activities were measured.

2. *Spectrophotometric Determination of the Inhibition of Copper-Catalyzed Oxidation of Epinephrine.* Lavollay (11) first employed this technique for measuring the inhibitory effects of "vitamin P"-like substances on the autoxidation of epinephrine. The present method is a refinement and extension of his technique.

One of the first steps in the oxidation of epinephrine is the formation of the red adrenochrome. Pure adrenochrome[5] was found to have an absorption maximum at 465 mμ. Rutin and many of the other substances tested, had maxima at or below 400. It was found that by selecting a band at 525, the red adrenochrome could be determined best in the presence of the yellow-colored inhibitors since the least superimposition of absorption occurred at 525. Substances with absorption maxima too near that of adrenochrome could not be studied (see table III). At 525 mμ adrenochrome obeyed Beer's law fairly well up to concentrations slightly less than 0.01 per cent.

After considerable study of temperature and pH optima, oxygenation time and rate, various buffers, epinephrine concentration and copper ion types and concentrations, it was found that epinephrine hydrochloride, 10 mgm. per 100 cc., in 0.1 to 0.2 molar Sørensen phosphate buffer at pH 7.3, was practically 100 per cent oxidized to adrenochrome, without melanizing, in the presence of 5 mgm. per cent $CuSO_4$ at 37.5°C. with vigorous oxygenation for 15 minutes. Oxygenation was critical and a very rapid rate of bubbling was found to give best results. Hydrogen peroxide oxidation was not studied. The oxygenation was performed by bubbling the gas from a pressure manifold through glass tubes immersed in the solution. The tubes were turned up at the ends to ensure free flow of gas.

The solutions were placed in 20 x 180 mm. test tubes. A total volume of 10 cc. solution was used, containing 0.1 to 0.2 molar Sørensen phosphate buffer at pH 7.3, 1.0 mgm. of epinephrine hydrochloride (in 0.1 cc. aqueous solution unstabilized epinephrine hydrochloride), and 0.5 mgm. of $CuSO_4$. The inhibitors were added as phosphate buffer solutions or in warm 10 per cent propylene glycol solution, it having been previously established that the amounts of propylene glycol used were in themselves not inhibitory. Everything but the epinephrine was added to a tube, which then was allowed to come to the temperature of the constant temperature water bath, and the oxygenation was started just before adding the epinephrine. Exactly 15 minutes after adding the epinephrine, the tube was read in the Coleman spectrophotometer at 525 mμ., using as a blank the same concentration of inhibitor without epinephrine. Concentrations of inhibitor were varied from 10 mgm. per cent up to concentrations giving 100 per cent inhibition, or if less active, to the limits of solubility (turbidity).

The per cent inhibition was obtained by running an epinephrine control without inhibi-

---

[5] Supplied by Hoffmann-La Roche, Inc.

tor, and dividing its log transmission into that for the tubes containing inhibitor These values for per cent inhibition then were plotted against concentration of inhibitor, and the value giving 50 per cent inhibition was determined from the graphs, as shown in figure 3 This value then was compared with the same value for rutin in order to express the activities in rutin units, both on a weight basis, and where the molecular weight was known, on a mol equivalent basis One "rutin unit" (r u) implies the same activity as rutin two units, twice the activity and so forth To give some idea of the molecular ratio needed for inhibition by the colorimetric method, 10 mgm of rutin completely inhibited the oxidation of 1 mgm of epinephrine hydrochloride under the experimental conditions employed, this is equivalent to 4 mol equivalents of rutin for every mol of epinephrine

Because of the insolubility of some of the flavonoid compounds in the phosphate buffer, many other solvents were tried, including methyl ethyl, n propyl and iso propyl alcohols, pyridine, ethyl acetate, propylene glycol, diethylene glycol, acetone, diacetin, formamide and dioxane These were mixed with various volumes of different buffers appropriate for pH 7 3 All the solvents, except pyridine, n propyl and iso propyl alcohol and dioxane, inhibited the oxidation of epinephrine to adrenochrome

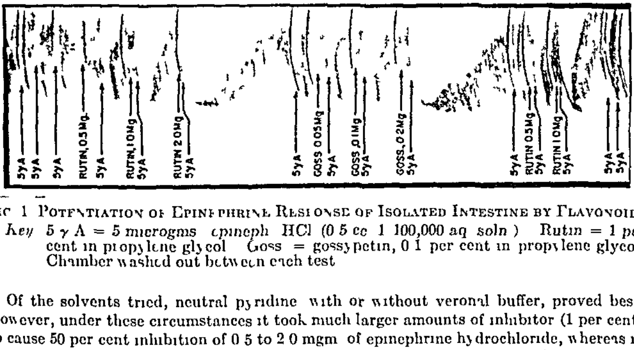


FIG 1 POTENTIATION OF EPINEPHRINE RESPONSE OF ISOLATED INTESTINE BY FLAVONOIDS
Key 5 γ A = 5 *microgms epineph* HCl (0 5 cc 1 100,000 *aq soln*) Rutin = 1 per cent in propylene glycol Goss = gossypetin, 0 1 per cent in propylene glycol Chamber washed out between each test

Of the solvents tried, neutral pyridine with or without veronal buffer, proved best However, under these circumstances it took much larger amounts of inhibitor (1 per cent) to cause 50 per cent inhibition of 0 5 to 2 0 mgm of epinephrine hydrochloride, whereas in the original aqueous phosphate buffer (when solubility was not the limiting factor), a concentration of only 10-25 mgm per 100 cc was necessary

It was concluded that solvents other than water are not suitable for the spectrophotometric determination of the inhibition by flavonoid compounds of the metal catalyzed oxidation of epinephrine *in vitro* Hence, the intestinal segment assay with its greater sensitivity to epinephrine, remains the assay of choice with compounds of low solubility or with those which have absorption maxima too near that of adrenochrome

RESULTS As an example of the methods and results of the intestinal segment assay of epinephrine potentiation, Exp 37 is given in some detail

Figure 1 illustrates a kymograph record taken from this experiment, in which the response to epinephrine alone and in the presence of increasing amounts of rutin and gossypetin are shown The half-recovery times in millimeters were measured from such records, and tabulated in terms of the number of times the response to epinephrine alone was potentiated Table I lists the results of these individual measurements and their averages, for the 8 compounds tested in

TABLE I

Epinephrine enhancement measurements from kymograph records. Exp. 37, 12/15/47

4 Gut segments (Nos. in parentheses indicate segment No.).

Total no. of control epinephrine tests (10 micrograms) = 127.

Prolongation of half-recovery times over that of Epinephrine alone

MGM /85 CC.	0.1	0.15	0.2	0.25	0.5	1.0	2.0	3.0	5.0
Rutin					2.4(1) 2.5(2) 2.7(3) 4.8(4)	3.0(1) 1.8(1) 9.7(1) 3.4(1) 3.3(2) 3.2(2) 3.9(2) 2.5(2) 5.2(3) 3.1(3) 3.5(3) 2.2(3) 3.7(4) 3.8(4) 9.7(4) 5.2(4)	3.5(1) 13.2(1) 9.5(1) 5.7(2) 8.2(2) 4.9(2) 6.5(3) 6.9(3) 4.6(3) 7.7(4) 15.8(4) 10.3(4)	7.6(1) 13.5(1) 8.4(2) 6.3(2) 13.1(3) 11.9(3) 8.7(4) 15.5(4)	12.7(2) 23.6(4)
Av.					3.1	4.2	8.1	10.6	18.1
Butein	0.9(1) 0.9(2) 2.9(3) 1.9(4)			2.2(1) 3.1(2) 5.0(3) 3.7(4)	7.5(1) 9.4(2) 14.2(3) 11.5(4)	Toxic*			
Av.	1.6			3.5	10.6				
Quercetin	2.3(1) 1.6(2) 1.9(3) 2.3(4)	5.0(1) 2.3(2) 2.5(3) 3.8(4)	4.1(2) 6.5(3)	7.2(1) 6.1(2) 6.9(3) 9.2(4)	37.2(1) 25.4(2) 29.2(3) 44.5(4)	Toxic			
Av.	2.0	3.4	5.3	7.3	34.1				
2′,3,4-trihydroxy-chalcone	3.4(1) 2.4(2) 1.5(3) 9.7(4)			11.5(1) 7.4(2) 7.3(3) 16.5(4)	37.2(1) 23.0(2) 20.8(3) 62.0(4)	Toxic			
Av. . . .	4.2			10.7	35.7				
Gossypetin	4.7(1) 2.5(2) 1.5(3) 3.2(4)			22.3(1) 16.8(2) 25.4(3) 23.9(4)	63.6(1) 36.9(2) 76.2(3) 70.0(4)	Toxic			

TABLE 1—*Continued*

MGM./85 CC.	0.1	0 15	0 2	0.25	0 5	1 0	2 0	3 0	5 0
Av	3 0			22 1	61 7				
Gossypin	1 2(1)			1 7(1)	6 6(1) 4 7(2) 3 5(3) 8 1(4)	14 3(1) 10 3(2) 13 1(3) 16 1(4)			
Av	*1 0*			*1 7*	*5 7*	*13 4*			
Gossypitrin				1 9(1) 1 3(3) 2 6(4)	7 8(1) 3 8(3) 11 6(4)	18 9(1) 14 6(3) 23 8(4)	Toxic		
Av				1 9	7 3	17 5			
3,3',4' trihydroxy flavone	3 2(1) 1 9(3) 4 7(4)	8 5(1) 5 3(2) 7 7(3) 11 9(4)	29 1(1) 16 8(2) 26 1(3) 29 0(4)	Toxic					
Av	3 2	8 3	25 2						

* Decrease in rhythmic contractions and tone before addition of epinephrine, or no recovery after addition of epinephrine

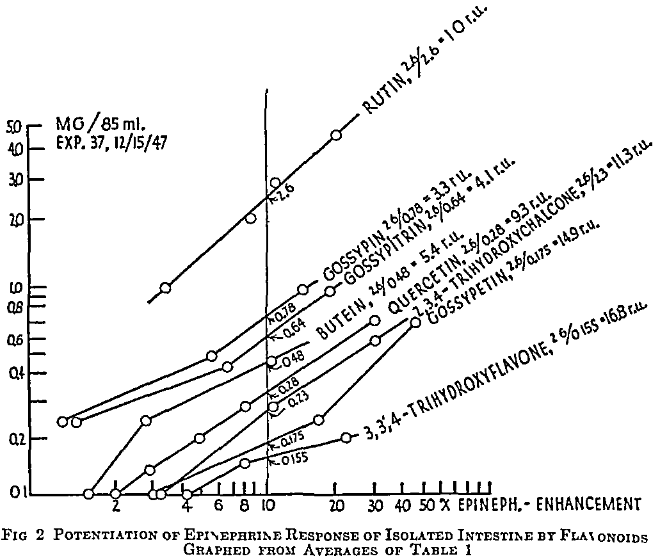


FIG 2 POTENTIATION OF EPINEPHRINE RESPONSE OF ISOLATED INTESTINE BY FLAVONOIDS GRAPHED FROM AVERAGES OF TABLE 1

TABLE II

Summary of epinephrine potentiation activities

COMPOUNDS TESTED	MOL WT.	ACTIVITY (RUTIN UNITS)				"VIT P" ACTIVITY (FROM LIT.)
		Gut Assay		Colorimetric		
		Wt. basis	Mol. basis	Wt. basis	Mol. basis	
1. Gossypetin[p]	318	16.0	8.5			
2. 3,3′,4′-trihydroxyflavone	270	15.5	7.0			
3. Quercetin sulfonic acid[q]	384	10.5	6.5+			
4. 8-hydroxyquinoline	145	26.0	6.0+			
5. Quercetin[q]	302	10.0	5.0+			+[a]
6. 2′,3,4-trihydroxychalcone	256	10.5	4.5			
7. Glutathione	307	9.0	4.5	1.39	0.70	
8. Na-diethyldithiocarbamate	171	12.5	3.5			
9. 3′,4′-dihydroxyflavone	254	8.0	3.5−			
10. Cysteine-HCl	158	11.0	3.0−	3.12	0.80	+[b]
11. Gossypin[p]	480	3.5	3.0−			
12. Gossypitrin[p]	480	3.5	3.0−			
13. Butein	272	6.0	3.0			
14. Quercetagetin[p]	318	4.0	2.0			
15. Esculetin	178	6.5	2.0			+[c]
16. Pyrogallol	126	8.0	1.5+			+[b]
17. 3,4-dihydroxychalcone	240	3.5	1.5−			
18. 2,3-dithiolpropanol (BAL)	124	6.0	1.0+	3.12	0.63	
19. Cyanin-Cl	646	1.0	1.0			
20. Rutin (unit standard)	610	1.0	1.0	1.0	1.0	+[e]
21. Leptosin	462	1.0	1.0−			
22. Nordihydroguaiaretic acid		<1.0	<1.0 (toxic)			
23. 3′,4′-dihydroxyflavanone	255	2.0	0.5+			
24. Epimerized *d*-catechin[r,s]	290	1.5−	0.5+			+[d]
25. *d*-catechin	290	1.0	0.5			−[d]
26. *l*-epicatechin[p,t]	290	1.0	0.5			−[d]
27. 3-hydroxy-3′,4′-dimethoxyflavone	398	1.0	0.5			
28. Chlorogenic acid	254	1.0+	0.5			
29. 2′,3′,4′,3,4-pentahydroxychalcone	303	1.0	0.5			
30. 5-hydroxyflavone	240	1.0	0.5 (toxic)			
31. Ascorbic acid	176	1.5	0.5	0.54[w]	0.15	±[f]
32. Xanthorhamnetin[q]	770	0.5	0.5−			+[g]
33. "Citrin" (various, incl. Szent-Gyorgyi)[t]		0.2–0.7	?	0.2–0.7	?	±[h]
34. Eriodictyol	288	0.5	Neglig.			+[i]
35. 4,4′-dihydroxychalcone	240	0.5+	Neglig.			
36. Disalicylalethylenediimine	268	0.5	Neglig.			
37. 3,4,7,3′4′-pentahydroxyflavanone[t]	303	0.5−	Neglig.			
38. Hesperetin[t]	202	0.5−	Neglig.			±[i]
39. 7,8,3′,4′-tetrahydroxyflavanone	287	0.5−	Neglig.			
40. Dihydroesculetin	180	0.5−	Neglig.			
41. Phloretin		Neglig.				±
42. Disalicylal-*o*-phenylenediimine		Neglig.				

TABLE II—*Continued*

COMPOUNDS TESTED	MOL. WT	ACTIVITY (RUTIN UNITS)				"VIT P" ACTIVITY (FROM LIT)[a]
		Gut Assay		Colorimetric		
		Wt. basis	Mol basis	Wt. basis	Mol basis	
43 Esculin		Neglig		Neglig		+[e]
44 Rutin acid phthalate		Neglig		Neglig		
45 Rutin acid succinate		Neglig		Neglig		
46 3,4′ dihydroxy 4 methoxychalcone-4′ glucoside[r]		Neglig				+[l]
47 Na α tocopherol phosphate		Neglig				+[m]
48 2′ hydroxychalcone		0	0			
49 2′,4′,6′,3 4 pentamethoxychalcone		0	0			
50 Hesperidin[t]		0	0			+[n]
51 Hesperidin acid phthalate		0	0	0	0	
52 Hesperidin acid succinate		0	0	0	0	
53 "Methylated hesperidin chalcone"[t]		0	0	0	0	
54 "Acetylated hesperidin chalcone"[t]		0	0	0	0	
55 "Hesperidin 3′ ethyl carbonate"[u]		0	0	0	0	−[o]
56 Naringin		0	0	0	0	
57 o hydroxyacetophenone		0	0			
58 4 methoxy 3 2′ 4′ 6′ tetrahydroxy chalcone 4′ glucoside[r]		0	0			+[l]
59 4′ aminochalcone glucoside[r]		0	0			+[l]
60 3,4′ dihydroxy 4 methoxy chalcone 4′ phosphate (disodium salt)[r]		0	0			+[l]
61 Butrin[p]		0	0			
62 Inositol		—	—	0	0	
63 Pomiferin[v]			Too insol			
64 Gossypol acetate			Too insol			
65 Catechol		Accels	oxid	Accels	oxid	−[b]
66 Resorcinol		—	—	Accels	oxid	
67 Na bisulfite		—	—	1 78	0 34	
68 Hydroquinone		—	—	Accels	oxid	−[b]

Lit refs on "vit P" activity exclusive of clinical work (see Bibliography) [a] 40, 43, 47 [b] 44 [c] 16, 45 [d] 12-16 [e] 40 47, 49 [f] (+) = 44, (−) = all other work [g] 40 [h] (+) = 42, 46, 49, 51, 56, 57, (−) = 48, 52, 54, 58 [i] 29, 43 [j] (+) = 29, 43, (−) = 55 [k] (+) = 16, (−) = 55 [l] 46 [m] 29 [n] (+) = 41, 42, 43, 49, 50, (−) = 40, 55 [o] 53 [p] Prof T R Seshadri, Andhra Univ, Waltair, S India [q] S B Penick Co, N Y [r] Hoffmann LaRoche, Inc, Nutley, N J [s] Prof J Lavollay, Paris, France [t] Calif Fruit Growers Exchange, Los Angeles, Calif [u] Takeda Pharmaceutical Industries, Ltd, Osaka, Japan [v] Prof M L Wolfrom, Univ Ohio, Columbus, Ohio [w] Synergizes rutin approx 3 5X

Exp 37, including the rutin standard As an illustration of the necessity of running repeated epinephrine control responses throughout the experiment, 127 tests with epinephrine alone were run in this particular experiment

The averages were plotted on log-log paper as illustrated in figure 2 It is seen that the curves are parallel Of course, as the concentrations become smaller, the curves approach an asymptote, and are no longer parallel straight

lines. Rectangular coordinate graphs also show straight lines, originating from the origin.

From this graph, by taking either a given concentration or prolongation time, the activities relative to rutin as unity were obtained, as illustrated in the figure.

From some 50 such experiments, performed in a little over a year's time, and

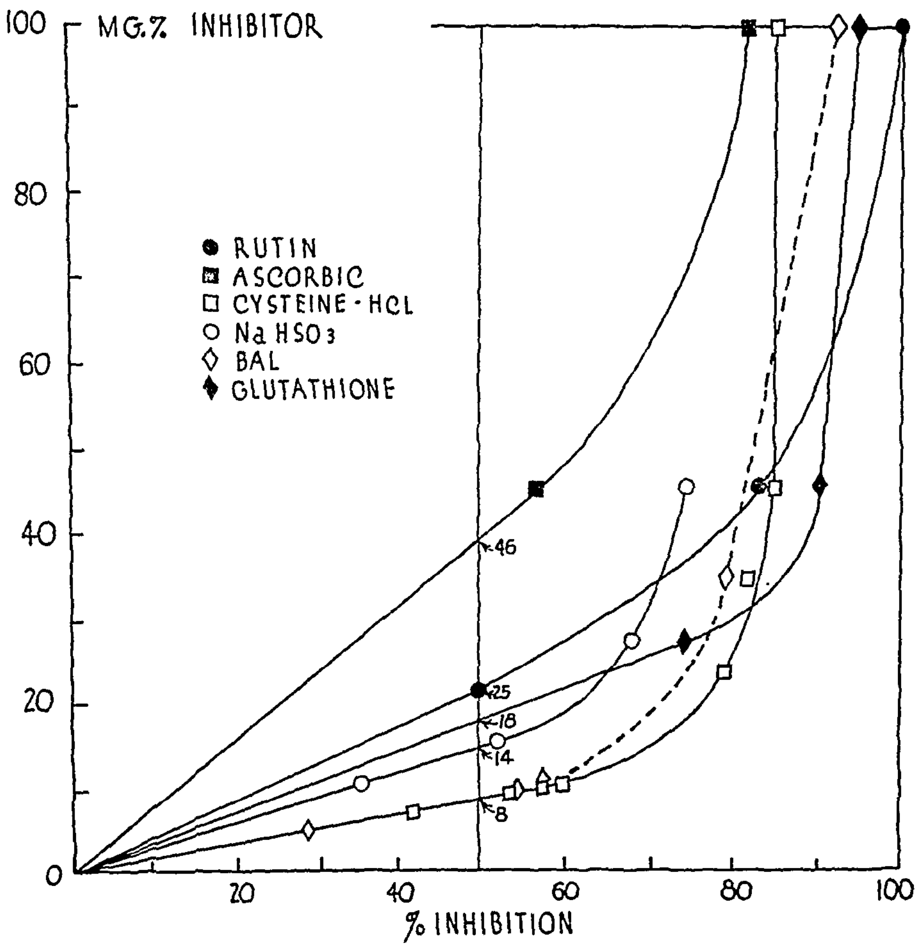


Fig. 3. Spectrophotometric Determination of Inhibition of Copper-catalyzed Auto-oxidation of Epinephrine

in several of which the same compounds were re-tested, the average activities in terms of rutin units on a weight basis and a mol basis were obtained for 68 compounds and are listed in table II. The actual values of "rutin units" are given to the nearest 0.5 r.u., as it was felt that smaller differences were not significant.

Figure 3 shows the results of some of the spectrophotometric measurements of the inhibition of the copper-catalyzed oxidation of epinephrine *in vitro*. The values in mgm. per cent of the various inhibitors at 50 per cent inhibition are

TABLE III

Spectrophotometric determination of the inhibition of copper catalyzed autoxidation of epinephrine

COMPOUND TESTED	MOL WT	MGM PER CENT CAUSING 50 PER CENT INHIBITION	ACTIVITY (RUTIN UNITS) Wt basis	ACTIVITY (RUTIN UNITS) Mol basis
1 Rutin[a]	610	25	1 0	1 00
2 Glutathione	317	18	1 4	0 70
3 Na bisulfite	104	14	1 8	0 34
4 Cysteine HCl	158	8	3 1	0 80
5 Dithiolpropanol ("BAL")	124	8	3 1	0 63
6 Ascorbic acid	176	46	0 5	0 15
7 Na rutin acid succinate[a]		350	0 07	
8 "Citrin"[b]		34	0 7	
9 Rutin + ascorbic acid	Activity of combination 3-4 times greater than either alone			
10 Na rutin acid phthalate			0	0
11 Na hesperidin acid phthalate			0	0
12 Methylated hesperidin chalcone [b]			0	0
13 Acetylated hesperidin chalcone [b]		Inactive	0	0
14 Esculin[c]			0	0
15 Naringin			0	0
16 Inositol			0	0
17 8 hydroxyquinoline				
18 Disalicylal o phenylenediimine				
19 Quercetin[a]				
20 Quercitrin				
21 Gossypetin[d]		Too insoluble		
22 Gossypol				
23 Eriodictyol				
24 Hesperidin				
25 Esculetin				
26 Phoretin				
27 Resorcinol		Accelerate oxidation		
28 Catechol				
29 Nordihydroguaiaretic acid				
30 Pyrogallol				
31 Hydroquinone				
32 Phloroglucinol				
33 Pyrogallic acid				
34 Gallic acid		Transmission maxima of oxidation products too close to 525 mμ to run		
35 Tannic acid				
36 Chalcones active by gut assay				
37 *d* catechin				
38 *l* epicatechin[b d]				
39 Epimerized *d* catechin[e f]				

[a] S B Penick & Co , N Y

[b] Calif Fruit Growers Exchange, Los Angeles and several fractions and purifications thereof

[c] Mercantile Export & Import Co , N Y

[d] Prof T R Seshadri, Andhra Univ , Waltair, S India

[e] Prof J Lavollay, Paris, France

[f] Hoffman LaRoche, Inc , Nutley, N J

indicated in figure 3. When divided into the value for rutin, the activities in terms of "rutin units" are obtained. Table III lists the results of such measurements for the 39 compounds tested. In addition, table II also lists the results, where the method was applicable.

The results of the gut assay experiments show that the most active compounds were gossypetin, quercetin, 2′,3,4-trihydroxychalcone 3,3′,4′-trihydroxyflavone and the well known metal complexers, 8-hydroxyquinoline and sodium-diethyldithiocarbamate.

Discussion. The results are in accord with those reported by Lavollay (36) who used the intestinal segment assay in a qualitative manner for studying the epinephrine-enhancing effects of a few flavonoids, since he reported quercetin > luteolin > rutin > morin > quercitrin, and negligible activity for hesperetin and naringin. Unfortunately his list of compounds tested was not extensive enough to allow him to draw any conclusions regarding the minimum structural requirements for highest activity.

Lavollay and his co-workers claimed a close parallelism between epinephrine potentiation by various flavonoids *in vitro* and *in vivo*, but as pointed out in the introduction of the present paper, there are conflicts in these claims of the French investigators (14, 24–29). Moreover, the present work does not show a parallelism between the spectrophotometric and the intestinal segment assay results. For this reason we did not make manometric studies *in vitro*.

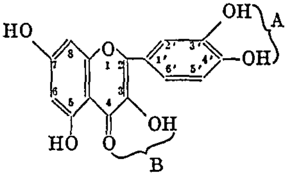
The French investigators (11–16) also claimed that of the 4 possible isomers of catechin, *d*-epicatechin was active in decreasing capillary fragility, whereas the others were not. The present results show no differences between *d*-catechin, *l*-epicatechin and epimerized *d*-catechin (a mixture of the 4 isomers, used by the French investigators as their source of *d*-epicatechin). It is interesting that our previous studies (59) also showed similar activity of these isomers as capillary bed vasoconstrictors.

A conspicuous result of the present study is the demonstration that there is no parallelism between the reported "vitamin P"-like activity of many of the substances studied and their epinephrine potentiation on isolated smooth muscle. This throws serious doubt on the epinephrine enhancement theory of the mechanism of action of "vitamin P"-like substances, and contrasts sharply with the conclusion of Torres (17) and others that the isolated intestinal segment is a suitable means of measuring "vitamin P" activity.

An examination of the active substances in the gut assay discloses that all of them possess in common one or more structural elements capable of complex formation with heavy metals. Moreover, for those pairs of substances possessing identical chelatogenic groupings the more active in each case is the one which would be expected to form the more stable complex. That chelation with copper actually takes place with these substances can be shown qualitatively by the formation of colored substances (in solution or as insoluble precipitates) when copper sulfate is added to their alcoholic solutions. Polarographic studies (60) have demonstrated clearly that complexes are formed, as shown by the disap-

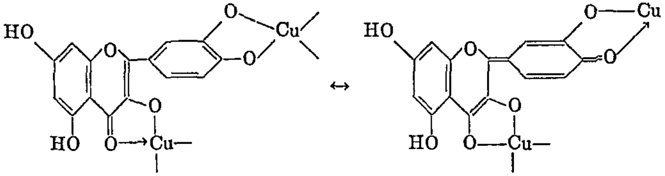
pearance or diminution of the normal copper wave and the appearance of a new wave at a more negative half-wave potential in mixtures containing copper sulfate and a number of the compounds shown to be active in the gut assay. 2'-hydroxychalcone, which contains a potential chelate-forming grouping, is devoid of activity in the gut assay and shows no indication of complex formation when studied polarographically. Recently, some of the copper-flavonoid complexes have been purified and the empirical formulae obtained from their elementary analyses were found to coincide with the calculated.

The greater (5 ×) activity of quercetin compared with rutin, and other observations, led to the conclusion that in the flavonoid compounds the important complex-forming elements were the 3',4'-dihydroxy grouping and the 3-hydroxy-4-keto grouping, shown as A and B in the formula for quercetin (I) below:



I

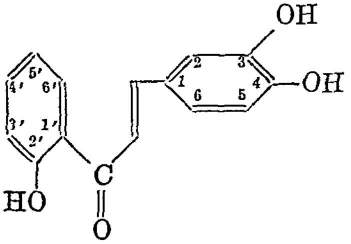
The importance of the *flavone* as contrasted with the *flavanone* structure, can be ascribed to the stabilization of the complex II by contributions to the resonance hybrid from forms such as III (note: the participation of other chelatogenic groupings in further combination with the copper atom is indicated but not shown in detail):



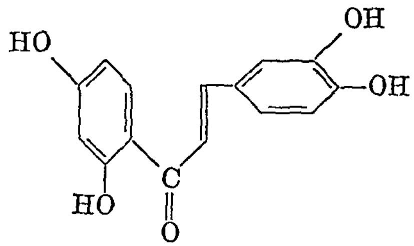
II III

From considerations such as these, it was predicted early in the work that 3,3',4'-trihydroxyflavone would form a complex stabilized to a greater degree (with respect to the un-complexed compound) than quercetin, since in the latter substance resonance stabilization of the un-complexed substance would involve not only the 3- and 3',4'-hydroxyl groups, but those in the *benzo* ring as well. The results confirmed this prediction, 3,3',4'-trihydroxyflavone being considered more active than quercetin. Similar arguments led to the study of 2',3,4-tri-

hydroxychalcone (IV); this was found to be more active than butein (V) from which it differs only in the absence of a non-chelatogenic hydroxyl group.

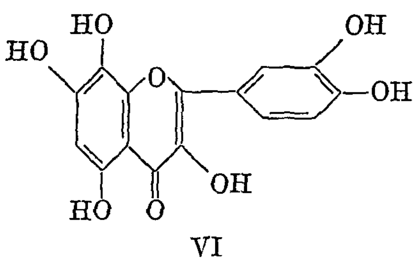


IV



V

These results, along with the occurence of high activity in this test of 8-hydroxyquinoline, support the view that these substances prolong the action of epinephrine by protecting it from copper-catalyzed oxidative destruction. This concept is not entirely novel, since Lavollay's early studies led him to a similar view, which, however, differed somewhat in detail from that which we are inclined to adopt. Lavollay suggested that compounds containing the catechol nucleus protected epinephrine from destruction by entering into a complex composed of epinephrine + copper + inhibitor, within which the protective substance was preferentially oxidized. That this mechanism may still be accepted in certain cases is shown by the high activity of gossypetin (VI) and the *acceleration* of epinephrine destruction by catechol (also noted by Lavollay).



VI

In gossypetin the 5,7,8-trihydroxy grouping represents a readily autoxidizable hydroquinone-like structure and may exert a direct anti-oxidant effect in protecting epinephrine against oxidation. In the cases of most of the other flavonoid substances tested, however, it is probable that their protective action results from an immobilization of the catalytic copper in the bath fluid. This conclusion is supported by the wide range of activity of that group of flavones which contain the catechol nucleus but which differ in other details, and by the protective action of such substances as 8-hydroquinoline and 5-hydroxyflavone, which would not be expected to act by the mechanism proposed by Lavollay.

The wide differences in activity shown by substances having identical chelatogenic groupings but differing in solubility characteristics (e.g., such pairs as gossypin and quercetin, rutin and xanthorhamnetin) supports this view, since in such pairs, the compound which is the more soluble exerts the lesser protective

effect on epinephrine This is in accord with what would be expected, a less soluble complex being more effective in removing copper from the medium

The possibility remains that in some cases compounds which prolong the effect of epinephrine on the gut segment may actually penetrate to the tissue sites at which the epinephrine acts, and may exert an effect by interaction with some enzyme system, the normal role of which is to destroy epinephrine That the compounds tested can penetrate into the tissue is indicated by the fact that many of them are toxic to the gut segment when used in sufficiently high concentrations It is our belief, however, that the major part of the effects observed occur in the bath fluid

The results of Bacq and others, who found enhancement *in vivo* of epinephrine mediated responses by such substances as catechol and pyrogallol, suggest that certain compounds of the kind used in this study might be capable of enhancing epinephrine responses in the intact organism by action at tissue sites (see 22) It is not possible, on the basis of our results, to regard the effects *in vivo* observed by Bacq, and the gut segment responses studied in this work as being manifestations of a common type of action

In connection with the theory of Lavollay that "vitamin P"-like compounds may enhance and prolong the action of epinephrine and sympathin *in vivo*, we have observed no effect of such substances on the magnitude or duration of the response of rabbit intestine to faradic stimulation of the inhibitory mesenteric nerve, using the preparation described by Hendricks and Thienes (61) The compounds tested were rutin, *l* epicatechin, epimerized *d* catechin, 2′,3,4 trihydroxychalcone, ascorbic acid, rutin + ascorbic acid, esculetin + ascorbic acid, sodium diethyldithiocarbamate, and BAL

In addition, we have observed no effects of various "vitamin P" like substances intravenously administered, on blood sugar[6] levels or upon magnitude or duration of epinephrine hyperglycemia in 12 hour fasted, standardized rabbits

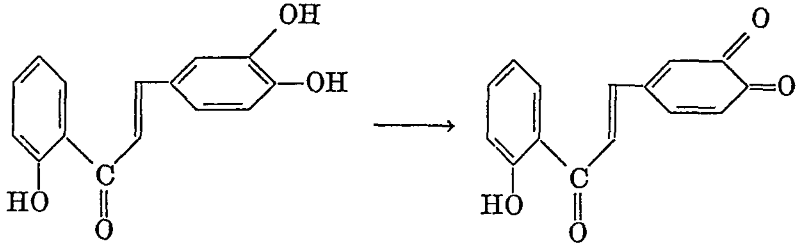
Lastly, we have shown that although epinephrine very effectively prevents

[6] A special blood sugar method had to be developed to remove "vitamin P" like substances from the blood, since they are reducing substances It was found that the ferric hydroxide blood precipitation method effectively removed the flavonoids used The method was modified from Steiner, Urban and West (62), using an excess of ferric sulfate and barium carbonate Sugars were determined in the protein free filtrates by the ceric sulfate method of Miller and Van Slyke (63) The compounds examined were administered intravenously in doses from 20 to 100 mgm /kgm alone or simultaneously with epinephrine hydrochloride, 0 1–0 25 mgm /kgm subcutaneously, and included rutin, sodium rutin acid succinate, xanthorhamnetin, rhamnetin, *d* catechin, 'methylated hesperidin chalcone", and quercetin sodium sulfonate Heart puncture blood samples were analyzed at different intervals for several hours before and after the injections

In addition, since the administration of metallic salts, especially of copper salts, has been reported to inhibit epinephrine hyperglycemia (64, 65, 66, 67), it was thought that the "vitamin P" like substances might antagonize this inhibition because of their metal chelating properties, but we were not able to demonstrate any such effect of copper sulfate, 1 0 mgm /kgm , or of copper glycinate, 5 mgm /kgm , administered intravenously 10 minutes prior to epinephrine hydrochloride, 0 1 mgm /kgm , injected subcutaneously into 12 hour fasted, standardized rabbits

angioneurotic-like edema in atopic eggwhite sensitivity in the rat (68, 69, 70), "vitamin P"-like compounds do not potentiate this epinephrine effect.[7]

Bartlett (71, 72) has shown that 2′,3,4-trihydroxychalcone (IV) which is highly active in the gut assay, inhibits succinoxidase, and that this action probably results from its oxidation to the ortho-quinone (VII),



IV VII

which then exerted an inhibitory action such as has been observed with other quinones. To suggest that those substances active in the gut segment assay and containing the catechol nucleus, act *solely* by a similar inhibition of some quinone-sensitive enzyme in the muscle is not in accord with the high activity of such substances as 8-hydroxyquinoline and the low activity of such sensitive hydroquinones as dihydroesculetin and eriodictyol, but should be kept in mind.

The mechanism of action of the flavonoid substances on the inhibition of hyaluronidase *in vitro*, studied by Bieler and Martin (73, 74) cannot be explained by quinoid inhibition (75) since some of the compounds they used (hesperidin and its derivatives) do not contain the unsubstituted *o*-dihydroxy grouping necessary for quinone formation.[8] It is possible that these substances may act by metal chelation of a heavy metal necessary for hyaluronidase action, to form a more stable hesperidin-metal complex, although the presence of a heavy metal as a necessary component of this enzyme to our knowledge has not yet been established.

[7] The effective dose of epinephrine was 75 to 100 micrograms/kgm. administered subcutaneously or intramuscularly simultaneously with or 30 minutes after the intraperitoneal injection of 0.5 to 1.0 cc. of raw eggwhite. The compounds tested were given by all possible routes of administration (intra-peritoneal injection of some of the drugs interfered with eggwhite absorption), at various times relative to the eggwhite injections, and in doses varying from 10 to 200 mgm./kgm. The compounds tested were: rutin, various "Citrins," "methylated hesperidin chalcone", butein, 2′,3,4-trihydroxychalcone, coreopsin (soluble 4′-glucoside of butein), epimerized *d*-catechin, esculetin, sodium salt of esculetin-4-carboxylic acid, quercetin, sodium salt of quercetin sulfonic acid, 5-chlorooxine, sodium diethyldithiocarbamate, 8-hydroxyquinoline and dithiolpropanol (BAL). The results were judged by the incidence and degree of edema of the face and paws. Nearly 1000 rats were used in this and related studies.

[8] We also found that several parenterally administered flavonoids inhibit the spreading effect of hyaluronidase *in vivo*, but massive doses were required (76). An unsubstituted *o*-dihydroxy grouping in the phenyl ring was essential for this action and only those compounds listed in table II as having high activity in the gut assay were effective. Levitan (77) recently has confirmed this, in the case of rutin.

SUMMARY

1 An isolated smooth muscle preparation is described for testing substances potentiating the action of epinephrine under these circumstances

2 Some 70 compounds were examined for the relation between molecular structure and activity of flavonoid like ("vit P" like) substances on this epinephrine effect

3 The minimum molecular structure essential for high activity was predicted, synthesized and confirmed to have such high activity It is 3,3',4'-trihydroxyflavone and has an activity about 16 times that of rutin, on a weight basis

4 The epinephrine-potentiating effect of the compounds is due chiefly to metal chelation, although this is not the only possible mechanism

5 The presence of an unsubstituted *ortho* dihydroxybenzene nucleus, while present in many of the more active compounds, also is not a structural necessity

6 A spectrophotometric method is described for measurement of the copper catalyzed oxidation of epinephrine to one of its red oxidation products, such as adrenochrome 36 compounds were tested by this method

7 The activity series obtained by the isolated smooth muscle assay method does not coincide with that by the spectrophotometric method

8 The activity series obtained by the isolated smooth muscle assay method does not correspond with capillary fragility decreasing ("vit P") activity as reported in the literature

9 "Vitamin P"-like substances have no effect on the magnitude or duration of response of intestine to stimulation of the inhibitory mesenteric nerve, upon blood sugar levels in the presence or absence of epinephrine hyperglycemia, nor upon the preventative effect of epinephrine upon eggwhite edema in rats

10 It is concluded that items 7 and 8 do not support the theory that "vit P"-like substances act in the intact organism by inhibiting the metal catalyzed oxidative destruction of epinephrine or sympathin and that the isolated intestinal segment response is not a valid assay method for "vitamin P" activity

11 The metal chelating and anti oxidant properties of the compounds studied may have other useful applications.

REFERENCES

1 BACQ, Z M Arch Internat Physiol , 42 340, 1936
2 BACQ, Z M Arch Internat Physiol , 44 15 1936
3 CLARK, A J , AND RAVENTOS, J Quart J Exper Physiol , 29 185, 1939
4 HERMANN H , VIAL J , AND CHATONNET, J Compt rend Soc de Biol , 139 114, 1945
5 KREITMAIR H Arch exper Path u Pharmakol , 176 326 1934
6 REISER, M Arch exper Path u Pharmakol , 190 389, 1938
7 STMOLNAR AND FRIDRICH Klin Wchnschr , 20 1039, 1941
8 MEIDINGER, F Arch Internat Pharmacodyn et de Therap , 72 264, 1946
9 DAOUD, K M , AND AYYADI, M A S Biochem J , 32 1424, 1938
10 LAWSON H C , AND THIENES, C H Fed Proc , 3 78, 1944
11 LAVOLLAY, J I L'autoxydation des diphénols, en particulier de l'adrénaline II Structure et rôle fonctionnel de la vitamine P, Herman and Cie, Paris, 1943
12 PARROT, J L , AND GALMICHE, P Bull Med , 59 413, 1945

13. GALMICHE, P.: "La résistance et la perméabilité des vaisseaux capillaires et la vitamine P," Librairie Le François, Paris, 1945.
14. PARROT, J. L.: Gaz. méd. de France, 53: 157, 1946.
15. GÉRO, E.: Bull. soc. sci. hyg. aliment., 34: 85, 1946.
16. JAVILLIER, M., AND LAVOLLAY, J.: Helv. Chim. Acta, 29: 1283, 1946.
17. TORRES, J. T.: Farm. Nueva, 11: 177, 1946.
18. VALDECASAS, F. G., AND MUSET, P. P.: Trab. Inst. Nac. Cienc. Med. Curso, II: 725, 1943–4.
19. MUSET, P. P.: "Introduccion al estudio de la vitamina P.," Miguel Servet, Barcelona, 1945.
20. VALDECASAS, F. G.: Rev. Esp. Fisiol., 1: 88, 1945.
21. WILSON, R. H., MORTAROTTI, T. G., AND DE EDS, F.: THIS JOURNAL, 90: 120, 1947.
22. FUHRMAN, F. A., AND CRISMON, J. M.: J. Clin. Investigat., 27: 364, 1948.
23. MERLINI, D.: Arch. Farmacol. Sper., 72: 167, 1941.
24. LAVOLLAY, J., AND NEUMANN, J.: Compt. rend. Acad. Sci., 212: 251, 1941.
25. LAVOLLAY, J.: Compt. rend. Soc. de Biol., 137: 23, 1943.
26. LAVOLLAY, J.: Ann. Pharm. France, 1: 39, 1943.
27. LAVOLLAY, J., AND PARROT, J. L.: Compt. rend. Acad. Sci., 215: 496, 1942.
28. PARROT, J. L.: Compt. rend. Soc. de Biol., 137: 171, 1943.
29. LAVOLLAY, J.: Personal Communications.
30. DEROUAUX, G., AND ROSKAM, J.: J. Physiol., 90: 2P, 1937.
31. DEROUAUX, G.: Arch. Internat. Pharmacodyn. et de Therap., 66: 202, 1941.
32. PARROT, J. L., AND GALMICHE, P.: Compt. rend Soc. de Biol., 139: 950, 1945.
33. UNGAR, G.: J. Physiol., 103: 18P, 1944.
34. UNGAR, G.: Endocrinol., 37: 329, 1945.
35. MUNRO, H. N., LAZARUS, S., AND BELL, G. H.: Nutrit. Abst. & Rev., 17: 291, 1947.
36. LAVOLLAY, J.: Compt. rend. Soc. de Biol., 135: 1193, 1941.
37. FRIEDENWALD, J. S., AND BUSCHKE, W.: Am. J. Physiol., 140: 367, 1943.
38. WILTSHIRE, M. O. P.: J. Physiol., 22: 88, 1931.
39. GRIFFITH, J. Q., JR., COUCH, J. F., AND LINDAUER, M. A.: Proc. Soc. Exper. Biol. & Med., 55: 228, 1944.
40. SEVIN, A.: Compt. rend. Acad. Sci., 216: 505, 1943.
41. BACHARACH, A. L., COATES, M. E., AND MIDDLETON, T. R.: Biochem. J., 36: 407, 1942.
42. BENSATH, A., ST. RUSZNYAK, AND SZENT-GYORGYI, A.: Nature, 138: 798, 1936; 139: 326, 1937.
43. RUSZNYAK, ST., AND BENKO, A.: Klin. Wchnschr., 20: 1265, 1941.
44. LAVOLLAY, J.: Compt. rend. Soc. de Biol., 138: 801, 1944.
45. LAVOLLAY, J., AND SEVESTRE, J.: Compt. rend. Soc. de Biol., 139: 270, 1945.
46. HUGHES, E. G., AND PARKES, M. W.: E. C. Barell Jubilee Volume (Hoffman-LaRoche, Inc.) p. 216, F. Reinhardt, Basle, 1946.
47. AMBROSE, A. M.: Fed. Proc., 7: 202, 1948.
48. MATUSIS, I. I.: Bull. Biol. Med. Exp. USSR, 4: 481, 1937.
49. SCARBOROUGH, H.: Biochem. J., 39: 271, 1945.
50. BHAGVAT, K.: Indian J. Med. Res., 34: 87, 1946.
51. BOURNE, G. H.: Nature, 152: 659, 1943.
52. DETRICH, L. E., DUNN, M. S., MCNAMARA, W. L., AND HUBBARD, M. E.: J. Lab. Clin. Med., 25: 684, 1940.
53. HIRAMATSU, N.: Jap. J. Dermatol. Urol., 49: 286, 1941. (Translated from the Japanese by the Public Health & Welfare Section, SCAP, Tokyo; Biblio. Sci. & Ind. Repts., 10: 204, 1948—PB #92309, 47 pp. + tables, U. S. Dept. Commerce.)
54. MCHENRY, E. W., AND PERRY, H. M.: J. Nutrit., 12: 12P, 1940.
55. MOLL, TH., AND KUSSNER, W.: E. Merck's Jahrber., 51: 58, 1937.
56. RUSZNYAK, ST., AND BENKO, A.: Science, 94: 25, 1941.
57. ZACHO, E. E.: Acta Pathol. Microbiol. Skand., 16: 144, 1939.

58. Zilva, S. S.: Biochem. J., 31: 915, 1937.
59. Haley, T. J., Clark, W. G., and Geissman, T. A.: Proc. Soc. Exper. Biol. & Med., 65: 202, 1947.
60. Geissman, T. A., and Friess, S. L.: Unpublished work.
61. Hendricks, M. D., and Thienes, C. H.: Proc. Soc. Exper. Biol. & Med., 28: 993, 1931.
62. Steiner, A., Urban, F., and West, E. S.: J. Biol. Chem., 98: 289, 1932.
63. Miller, B. F., and Van Slyke, D. D.: J. Biol. Chem., 114: 583, 1936.
64. Schnetz, H.: Arch. exper. Path. u. Pharmakol., 178: 420, 1935.
65. Schwab, H.: Compt. rend. Acad. Sci., 207: 409, 1938.
66. Shimamura, M.: Folia Pharmacol. Japan, 26: 287, 1939.
67. Shkol'nik, M. I.: Byull. Eksptl. Biol. Med., 22: 27, 1946.
68. Léger, J., Masson, G., and Prado, J. L.: Proc. Soc. Exper. Biol. & Med., 64: 366, 1947.
69. Léger, J., and Masson, G.: Am. J. Med. Sci., 214: 305, 1947.
70. Léger, J., and Masson, G. M. C.: Ann. Allergy, 6: 131, 1948.
71. Bartlett, G. R.: Fed. Proc., 7: 6, 1948.
72. Bartlett, G. R.: This Journal, 93: 329, 1948.
73. Bieler, J. M., and Martin, G. J.: J. Biol. Chem., 171: 507, 1947.
74. Bieler, J. M., and Martin, G. J.: J. Biol. Chem., 174: 31, 1948.
75. Meyer, K., and Ragan, C.: Fed. Proc., 7: 173, 1948.
76. Clark, W. G.: Unpublished work.
77. Levitan, B. A.: Proc. Soc. Exper. Biol. & Med., 68: 566, 1948.

DEVELOPMENT OF RESISTANCE TO CHLORGUANIDE (*PALUDRINE*) DURING TREATMENT OF INFECTIONS WITH *PLASMODIUM CYNOMOLGI*[1]

L. H. SCHMIDT, CLARA SESLER GENTHER, ROCHELLE FRADKIN, AND WANDA SQUIRES

The Christ Hospital Institute of Medical Research, Cincinnati, Ohio

Received for publication November 16, 1948

During the course of studies on the activity of chlorguanide (*Paludrine*) against infections induced by inoculation of rhesus monkeys with the trophozoites of *Plasmodium cynomolgi*, it was observed that doses of this drug, which cured 94 per cent of all previously untreated infections, frequently failed to cure those infections which had originally been exposed to subcurative doses. Such unconventional responses were not an invariable occurrence when infections were first treated with subcurative doses of chlorguanide, but they did arise with sufficient frequency to require explanation. The possibilities of drug deterioration, defective drug absorption or degradation, and inadequate immune responses were considered, but, when submitted to critical consideration, did not provide satisfactory explanations. The most plausible possibility appeared to be that the *Plasmodium* had developed a tolerance for chlorguanide, a phenomenon which, as Bishop and Birkett (1), and Williamson and coworkers (2) were shortly to demonstrate, could take place during the treatment of *P. gallinaceum* infections in chicks. In order to test this explanation, a more systematic investigation was undertaken the next time the phenomenon, mentioned above, presented itself. This opportunity occurred in September 1947. The details and results of the studies carried out at that time are presented here.

EXPERIMENTAL. *A. General Procedures.* Young, sexually immature rhesus monkeys (*Macaca mulatta*), weighing 3 to 5 kgm., were used in all of the experiments described below. All of the animals had been imported recently from India and had not been used in any other experimental studies either in these laboratories or elsewhere.

The strain of *P. cynomolgi* used in these studies was obtained in June 1946, from Dr. Richard J. Porter, University of Michigan, Ann Arbor. It is our understanding (3) that this strain, which had been imported from India during the war years, was the one which Mulligan (4) had isolated from a cynomolgus monkey (*M. cynomolgus*) some ten years previously and that it had been maintained subsequent to isolation by frequent serial passage of trophozoites through rhesus monkeys. The precise history of the strain since its receipt in the United States is not known. However, after its admission to our laboratories and prior to its use in the first of the present experiments, the strain had undergone 28 serial trophozoite passages through rhesus monkeys. These passages, at approximately two-week intervals, were carried out by intravenous inoculation of from 50,000 to 500,000 trophozoites. Numerous chemotherapeutic experiments with trophozoite-induced infections have been performed since the strain was received here. All of these experiments indicate that the strain is entirely stable in its response to such antimalarial drugs as quinine, quin-

[1] This work was supported in part by a grant-in-aid from the United States Public Health Service.

acrine, chloroquine, oxychloroquine, and, with the exceptions noted in this report, to chlorguanide

The greatest portion of the work described in this report was carried out on trophozoite-induced infections Such infections were usually produced by the intravenous inoculation of 50,000 to 500,000 trophozoites After determination of the parasite density in the donor's blood, these inocula were obtained by a suitable dilution of heparinized blood in 0 85 per cent saline, the required dose of parasites being contained in 2 cc of diluted material

A few experiments with sporozoite induced infections were carried out Such infections were produced by the intravenous injection of sporozoite suspensions, prepared by grinding the thoraces of infected *Anopheles quadrimaculatus* in a 1 1 mixture of normal monkey serum and 0 85 per cent saline These mosquitoes had been fed 15 to 17 days previously on infected monkeys, and had been maintained since that time at a temperature of 27–28°C and a relative humidity of 70–80 per cent

All drug treatments were of the same pattern The daily doses of chlorguanide, or in later studies chloroquine, quinacrine, or pentaquine, were dissolved in 50 cc quantities of water, and were administered via stomach tube once daily for either 7 or 14 consecutive days Chlorguanide and quinacrine were administered as hydrochloride salts, chloroquine and pentaquine as the phosphates The dose of drug was always calculated in terms of base With few exceptions, drug treatments were given between 8 30 and 9 00 A M, immediately following the preparation of blood films for parasite examination

A single preparation of chlorguanide was used in all of the work described in this report This preparation was made in the laboratories of Imperial Chemical Industries, Ltd, and was made available through the courtesy of Dr D G Davey

Examinations of the blood for parasites were made by conventional thin and thick film procedures, using Giemsa stained preparations Such films were prepared from blood obtained from the marginal ear vein Parasite studies were made at least once daily (usually at 8–9 A M) during the period when the monkeys were receiving treatment and for at least a week after treatment ended or after thick films became negative After that time, single weekly or twice weekly examinations were made Parasite densities were determined on thin films as the number of parasites per 10,000 erythrocytes Estimations of the proportions of parasites of different stages were also made routinely The categories enumerated included early and late rings, mature trophozoites, schizonts, segmenters, and gametocytes

In order to determine whether infections in treated animals remained latent or were cured, an attempt was made to induce relapse through splenectomy This procedure was followed with all animals which had received chlorguanide or other medication, and whose blood had remained parasite free on thick film studies for 8 or more weeks after treatment If parasitemia did not result within 4 weeks after splenectomy, the infection was considered cured In work in this laboratory with some 800 monkeys, this criterion has proved valid in more than 99 per cent of the cases

B Activity of Chlorguanide against Infections with the Parent Strain of Plasmodium cynomolgi In order to appraise critically the reactions to chlorguanide set forth in later sections of this report, it is necessary to describe briefly those data which have been obtained in this laboratory with infections induced by trophozoites of the parent strain Such a summary dealing with the effectiveness of the drug against early primary attacks is presented in table 1

This table shows clearly the gradation in effectiveness of different doses of chlorguanide against infections with the parent strain Daily doses of 0 075 mgm per kgm were without curative effects although they always suppressed the parasitemia Doses of 0 15 and 0 3 mgm per kgm effected approximately 50 per cent cures, doses of 0 6 mgm per kgm effected more than 90 per cent cures, while larger doses than this were invariably curative In every one of

those cases not cured by chlorguanide, the parasitemia was reduced to a level where thick films were negative for at least 3 successive days. With the exception of 2 of these latter cases (one each on the 0.075 and 0.15 mgm. regimes), where parasites reappeared after 3 to 5 days, all of the animals had persistently negative thick films for periods of 10 to 36 days. Although the supporting data are not shown here, it should be stated that chlorguanide was equally effective against early primary and late primary attacks and against first relapses occurring subsequent to treatment with other antimalarial drugs. Additional studies have shown that doses of chlorguanide as small as 0.0188 mgm. per kgm. reduce the parasitemia significantly and in some instances abolish it temporarily.

A graphical summary of the effects of adequate chlorguanide treatment on the course of an infection with the parent strain is shown in figure 1. For purposes of comparison, the course of a typical untreated infection is also illustrated.

TABLE 1

Effectiveness of chlorguanide against infections induced with trophozoites of the parent strain of Plasmodium cynomolgi*

DAILY DOSE CHLORGUANIDE† MGM. BASE/KGM. BODY WEIGHT	NO. OF MONKEYS TREATED	RESULT OF TREATMENT	
		No. of relapses‡	No. of cures
0.075	4	4	0
0.15	12	6	6
0.3	12	5	7
0.6	17	1	16
1.25	8	0	8
2.5	4	0	4

* Data presented include results obtained in the treatment of early primary attacks only.

† Daily dose of drug administered for 7 consecutive days.

‡ The term 'relapse' has been used to indicate the reappearance of parasites in the blood following negative thick films on 3 or more successive days.

The data on the treated infection (Monkey 1414) show that treatment for 2 days with 0.16 mgm. per kgm. doses of chlorguanide effected a significant reduction in parsitemia. However, parasites in small numbers persisted in the blood throughout the entire 7 days of therapy and for 2 days after the end of treatment, even though this treatment was adequate for cure of the infection. This relatively slow response of the parasitemia is characteristic of the action of chlorguanide and, as will be pointed out later, is strikingly different in a number of respects from the rapid responses obtained with chloroquine, quinacrine, or quinine. The data on a typical untreated infection (Monkey 1546) show that the natural course of the disease is characterized by a primary attack of approximately 45 days duration and a series of 3 relapses, each of lesser intensity and length than the first attack. As is usual in untreated trophozoite-induced infections with this strain of *P. cynomolgi*, the disease was self-limiting in a period of about four months.

C. Development of Resistance to Chlorguanide during Treatment of Trophozoite-induced Infections. A systematic study of the problem of chlorguanide resistance was initiated when it was noted that the infection in Monkey 1373 had failed to respond satisfactorily to successive courses of treatment with chlorguanide at daily doses of 0.3, 0.3, 0.6, and 1.25 mgm. per kgm. At this point two plans were followed in the attempt to bring the strain of *P. cynomolgi* to the place where the parasites would produce an infection wholly indifferent to treatment with the maximum tolerated doses of the drug. The first of these plans involved continued treatment of the infection in Monkey 1373 with increasing

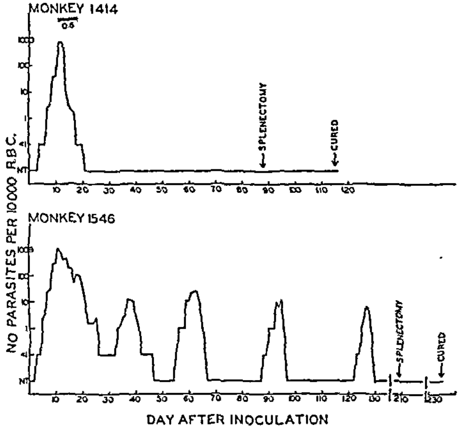


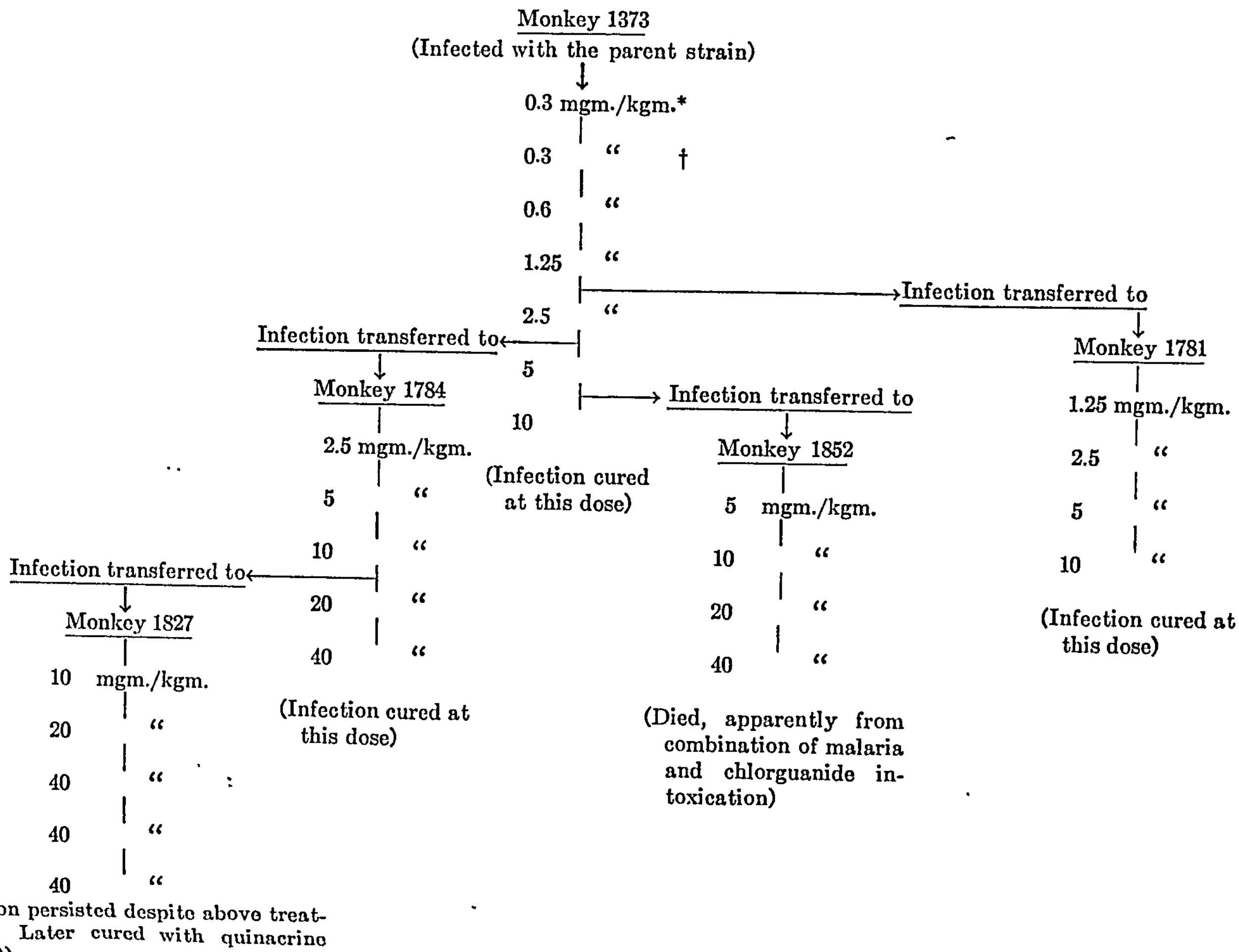
FIG. 1. THE EFFECT OF ADEQUATE TREATMENT WITH CHLORGUANIDE ON THE COURSE OF THE INFECTION INDUCED BY THE PARENT STRAIN OF *Plasmodium cynomolgi*

Monkey ... ily for 7 days as indicated (xxxxxxx). ... but less than 1 parasite per 10,000 e ... on study of 200 oil immersion fields.

doses of chlorguanide, the second, serial transfer of the infection from Monkey 1373 to clean monkeys and subsequent treatment of the infections in these animals with increasing doses of the drug. The form which this plan followed is outlined in figure 2.

As figure 2 shows, the infection in Monkey 1373 was treated successively with chlorguanide at daily doses of 0.3, 0.3, 0.6, 1.25, 2.5, 5, and 10 mgm. per kgm. During the last of these courses of treatment, parasites disappeared from the blood and did not reappear despite splenectomy; consequently, the animal was regarded as cured. Prior to this event, infected blood obtained from Monkey 1373 was used to inoculate 3 clean monkeys. The first of these animals, Monkey

FIG. 2. OUTLINE OF STEPS INVOLVED IN THE DEVELOPMENT OF A CHLORGUANIDE RESISTANT STRAIN OF *Plasmodium cynomolgi*



* Daily dose of drug administered for 7 consecutive days in all cases with one exception noted below.
† Daily dose of drug administered for 14

1781, was inoculated after the treatment of Monkey 1373 with 1.25 mgm. doses of chlorguanide. The infection in Monkey 1781 was treated with doses of 1.25 2.5, 5, and 10 mgm. per kgm., cure resulting after the last of these regimes. The third of the subinoculees, Monkey 1852, was inoculated subsequent to the treatment of Monkey 1373 with 5 mgm. doses of chlorguanide. Successive treatment of Monkey 1852 with doses of 5, 10, 20, and 40 mgm. per kgm. failed to eradicate the infection. Death occurred 2 days after the end of the treatment with 40 mgm. doses, probably the result of a combination of chlorguanide intoxication with the sequelae of persistent malaria. Numerous parasites were present in the blood of this animal at the time of death.

From the viewpoint of the objectives of the experiment, Monkey 1784, the second animal to receive blood from Monkey 1373, and its subinoculee, Monkey 1827, are probably the most important animals listed in figure 2. Monkey 1784 was infected after treatment of Monkey 1373 with chlorguanide at doses of 2.5 mgm. per kgm. The infection in Monkey 1784 was subsequently treated with doses of 2.5, 5, 10, 20, and 40 mgm. per kgm., the disease being eradicated by the last of these treatments. Subsequent to the treatment with 10 mgm. doses, the infection in Monkey 1784 was transferred to Monkey 1827. The infection in this latter animal was treated successively with chlorguanide at doses of 10, 20, 40, 40, and 40 mgm. per kgm. Despite repeated exposure to the 40 mgm. doses, which are maximum tolerated doses of chlorguanide (5), the infection persisted. Subsequent treatment with quinacrine, 10 mgm. per kgm. daily for 7 days, led to prompt eradication of the infection.

It seems fairly evident from the data presented in figure 2 that passage of the strain of *P. cynomolgi* through only 3 animals treated with successively increasing doses of chlorguanide resulted in a phenomenal change in the response of the parasite to the drug. A graphical summary of this alteration in response, as it occurred in Monkeys 1373, 1784, and 1827, is presented in figure 3. The data in this figure point to a stepwise acquisition of chlorguanide resistance by the parasite. Thus the infection in Monkey 1373 was initially affected very markedly by chlorguanide in doses of 0.3 mgm. per kgm., the parasitemia being reduced sharply to the point where thick films were negative. In contrast to this, the infection induced in Monkey 1784, a subinoculee from Monkey 1373 after the parasites in this latter animal had been exposed to chlorguanide for some 42 days, showed essentially no response to treatment with 2.5 mgm. doses; the infection in Monkey 1784 was affected significantly, however, by treatment with 10 and 20 mgm. per kgm. doses of chlorguanide and was ultimately cured by doses of 40 mgm. per kgm. In further contrast, the infection in Monkey 1827, a subinoculee from Monkey 1784, exhibited no significant response to 10 mgm. doses of chlorguanide and was not cured by repeated exposure to doses of 40 mgm. per kgm.

Final and conclusive proof of the development of chlorguanide resistance was obtained through an experiment in which the responses of infections induced by the parasites persisting in the blood of Monkey 1827 were compared with the responses of infections induced by the parent strain. Nine monkeys were used

in this experiment. Three of the animals were infected with the parent strain, 6 with the strain isolated from Monkey 1827. In all cases the infecting dose was approximately 400,000 erythrocytic parasites, injected intravenously. In all cases infections were patent the day after inoculation. Chlorguanide treatments were initiated during the ascending phase of the primary attack at a time when the infection involved from 1–5 per cent of the erythrocytes. The drug

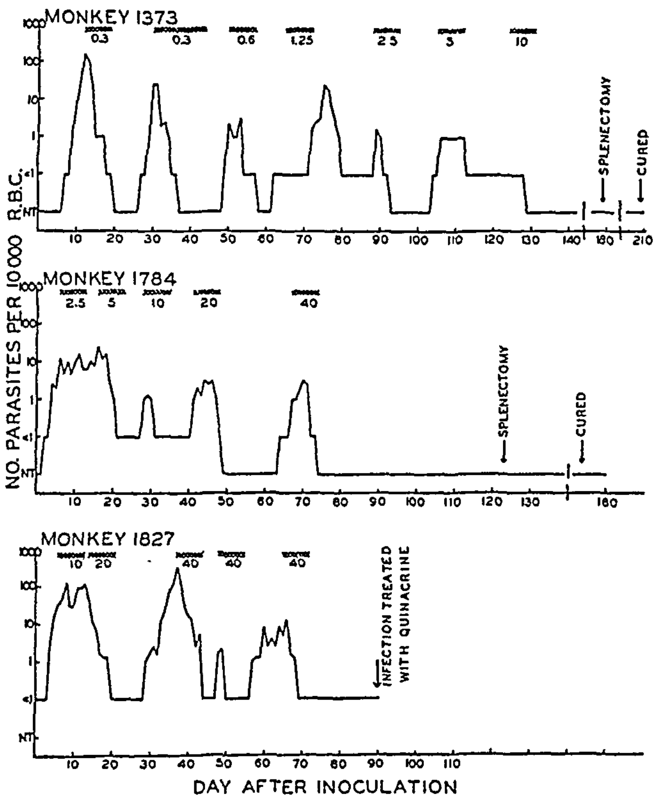


FIG. 3. THE CHANGES IN RESPONSE TO CHLORGUANIDE DURING DEVELOPMENT OF THE RESISTANT STRAIN

0.3–40 = daily dose of chlorguanide in mgm. per kgm. body weight. xxxxxxxx = days of chlorguanide treatment. <1 = positive thick film, but less than 1 parasite per 10,000 erythrocytes on thin film. NT = thick film negative on study of 200 oil immersion fields.

was administered via stomach tube in single daily doses, 0.6 mgm. per kgm. to the 3 monkeys infected with the parent strain, 0.6 mgm. per kgm. to 3 of the monkeys infected with the resistant strain, and 40 mgm. per kgm. to the remaining 3 animals infected with this latter strain. Studies of the numbers and distribution of the parasites and tests of cure were carried out as described in section A of this report. The results of the study have been summarized in tables 2 and 3.

As shown in table 2, the effectiveness of chlorguanide against infections with the resistant strain was markedly different from the activity of the drug against

infections with the parent strain. As would be expected, chlorguanide at daily doses of 0.6 mgm. per kgm. eradicated the disease in all 3 monkeys infected with the parent strain. Neither this dose nor the much greater one of 40 mgm. per kgm. cured any of the infections with the resistant strain. Not only were these doses ineffective in curing the latter infections, but they also failed to exert any significant effect on the parasitemias. Such reductions in parasite numbers as did occur differed little from some of the spontaneous reductions observed in untreated infections (cf. figure 1).

TABLE 2

Effectiveness of chlorguanide against infections with the trophozoites of the parent and resistant strains of Plasmodium cynomolgi

	DAILY DOSE	NUMBER PARASITES PER 10,000 RBC								REMARKS
Infections with parent strain										
1986	0.6	126	59	44	2	1	<1	<1	neg	Infection cured
1987	0.6	115	976	672	42	7	2	2	<1	Parasitemia abolished 3 days after end of treatment. Infection cured
1988	0.6	465	594	480	14	<1	<1	<1	<1	Parasitemia abolished 2 days after end of treatment. Infection cured
Infections with resistant strain										
1968	0.6	317	790	903	488	174	71	15	22	Infection persisted
1974	0.6	477	296	1869	742	139	51	122		Infection persisted
1975	0.6	146	552	588	504	168	136	112	198	Infection persisted
1976	40	186	146	432	415	233	148	26	20	Infection persisted
1977	40	135		1413	350	263	106	43	29	Infection persisted
1978	40	91		40	11	153	236	248	370	Infection persisted

Further evidence of the difference in the action of chlorguanide against infections with the two strains is shown in table 3. The data on Monkey 1988, typical of those obtained during chlorguanide treatment of an infection with the parent strain, show that reproduction of the parasites was stopped abruptly just prior to the occurrence of segmentation. Schizonts with numerous chromatin particles developed, but segmentation with merozoite formation did not occur. Within a period of 4 days, nearly all of these unsegmented schizonts were eliminated from the peripheral blood. The data on Monkeys 1975 and 1976 are in marked contrast to those just described. In these animals infected with the resistant strain, chlorguanide had no effect on the reproduction of the parasites. The cycles of growth, segmentation, and infection of new erythrocytes proceeded just as in untreated controls.

On the basis of these findings, the conclusion seems inescapable that serial exposure of the trophozoites of the parent strain to gradually increasing doses of

chlorguanide did lead to the development of resistance to this drug, a resistance of such degree that infections were essentially unaffected by the drug in daily doses of 40 mgm. per kgm., the maximum tolerated intake of this compound. When it is pointed out that chlorguanide in doses as small as 0.0188 mgm. per

TABLE 3

Effects of chlorguanide on asexual reproduction of the parent and resistant strain

MONKEY NUMBER	DAILY DOSE CHLORGUANIDE MGM. BASE/KGM. BODY WEIGHT	DAY OF TREATMENT	NUMBER OF PARASITES PER 10,000 RBC	PER CENT OF PARASITES AS				
				Early rings	Late rings	Mature trophozoites	Schizonts	Segmenters
Infections with parent strain								
1988	0.6	0	465	52	18			30
		1	594	7	79	3	11*	
		2	480			10	90*	
		3	14			29	71*	
		4	<1			Present		
		5	<1			Present		
		6	<1			Present		
		7	neg					
Infections with resistant strain								
1975	0.6	0	146	5	88	7		
		1	552	96				4
		2	588	7	93			
		3	504	68	1	2		29
		4	168	13	87			
		5	136	75		1		24
		6	112	10	90			
		7	198	83	1	2		14
1976	40	0	186	4	96			
		1	146	41		3		56
		2	432	4	96			
		3	415	34		1		65
		4	233	4	94	2		
		5	148	12	3	9		76
		6	26	8	89	3		
		7	20		15	10		75

* These schizonts were normal in appearance.

kgm. systematically stops reproduction and temporarily abolishes the parasitemia in infections with the parent strain, it becomes clear that more than a two thousandfold change in the response of the parasite to the drug had occurred during the development of the resistant strain.

It should be noted here that with respect to morphology, cyclic reproduction, and course of infection in the untreated monkey, the chlorguanide resistant strain of *P. cynomolgi* appears to be identical with the parent strain.

C. Transfer of Chlorguanide Resistance Through the Infected Mosquito. Whether chlorguanide resistance which developed during treatment of trophozoite-induced infections would be passed to the sexual forms of the parasite and carried through the infected mosquito was one of the most important questions arising in the above work. The following experiment was carried out to determine this point.

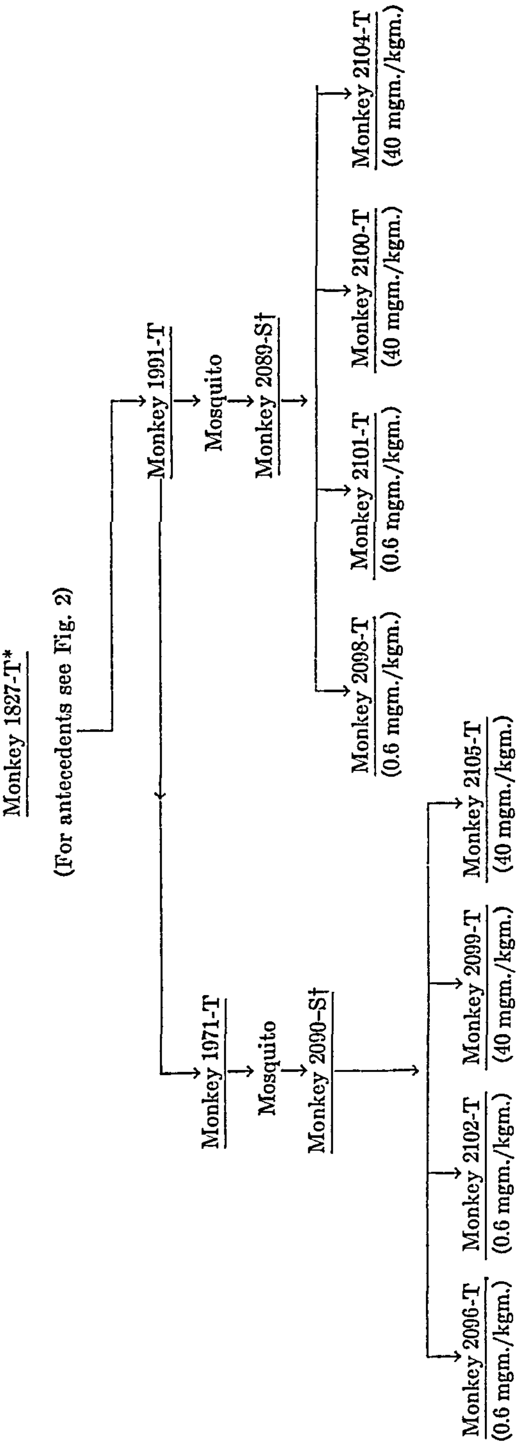
Monkey 1991 was inoculated with parasites obtained from the blood of Monkey 1827 subsequent to the final treatment of the latter animal with chlorguanide (cf. figure 2). When the parasitemia in Monkey 1991 was well developed, blood from this animal was transferred to Monkey 1971. Anopheline mosquitoes were allowed to feed on either Monkey 1971 or 1991. Highly infected groups of mosquitoes were obtained from both monkeys. The respective groups derived from the above animals were used to inoculate Monkey 2090 and Monkey 2089. These latter monkeys developed patent infections in 15 and 14 days respectively. Six days after the development of parasitemia, blood from Monkeys 2089 and 2090 was used to inoculate two groups of clean animals, 4 monkeys in each group; the infecting doses in these cases approximated 400,000 parasites. When the parasitemias in these latter animals reached appropriate levels (i.e., involving from 0.5 to 5 per cent of the erythrocytes), the infections from 2 monkeys of each group were treated with 0.6 mgm. per kgm. doses of chlorguanide while the remaining 2 were treated with doses of 40 mgm. per kgm. These doses were selected so that a single experiment would provide evidence either for the retention or loss of the resistant characteristic. Figure 4 contains an outline of the transfer procedure; the results of chlorguanide treatment are summarized in table 4.

The data presented in table 4 show that none of the infections was affected to a significant extent by treatment with chlorguanide at doses of 0.6 or 40 mgm. per kgm. Except for the unusual severity of the disease, resulting in death of 2 of the monkeys, the responses of these infections to chlorguanide were not different from the responses of those infections induced by parasites obtained directly from Monkey 1827 (cf. tables 2 and 4). It is clear, therefore, that the characteristic of chlorguanide resistance is transmitted through the infected mosquito. That this was not a chance happening is indicated by the fact that two separate mosquito passages derived from different monkeys yielded identical results.

D. Retention of the Resistant Characteristic during Serial Transfer of the Trophozoite-induced Infection. Subsequent to the development of the chlorguanide resistant strain of *P. cynomolgi* in Monkey 1827, the trophozoites of this strain were passed through untreated monkeys at approximately 3- to 4-week intervals. After 6 such passages, covering a period of 21 weeks, an experiment was carried out to determine whether a change in the resistant characteristic had occurred. Two monkeys were infected with parasites obtained from the 6th serial passage of the resistant strain. When the infections in these animals involved approximately 4 per cent of the erythrocytes, chlorguanide was administered to the respective monkeys in daily doses of 0.6 or 40 mgm. per kgm.

The results of this experiment will not be described in detail. It is sufficient

FIG. 4. OUTLINE OF STEPS INVOLVED IN THE TRANSFER OF THE CHLORGUANIDE RESISTANT STRAIN THROUGH THE MOSQUITO



* T = trophozoite-induced infection.

† S = sporozoite-induced infection.

to point out that the infections in the above monkeys were just as indifferent to treatment with chlorguanide as the infections with the freshly isolated resistant strain described earlier in this report (cf tables 2 and 4) It appears, therefore, that the resistant characteristic is a fairly well established property of the parasite

E Activities of Chloroquine, Quinacrine, and Pentaquine against Infections with the Chlorguanide Resistant Strain One of the practical questions arising from the above work concerns the effect which the acquisition of chlorguanide resistance might have on the response of the parasite to other antimalarial drugs For that reason, brief studies have been made of (a) the effectiveness of chloroquine and quinacrine against infections induced by the trophozoites of the chlorguanide resistant strain, and (b) the activity of pentaquine against infections induced by sporozoites derived from the resistant strain

TABLE 4

Effect of mosquito passage on the response of the resistant strain to chlorguanide

MONKEY NUMBER	DAILY DOSE CHLORGUANIDE MGM BASE/KGM BODY WEIGHT	NUMBER PARASITES PER 10 000 RBC								REMARKS
		At start of treatment	After day of treatment							
			1st	2nd	3rd	4th	5th	6th	7th	
Group infected with trophozoites from Monkey 2089										
2098	0 6	187	258	336	642	232	434	62	350	Infection persisted
2101	0 6	56	546	400	200	210	76	106	290	Infection persisted
2100	40	236	180	158	468	290	514	231	198	Infection persisted
2104	40	212	390	224	560	196	482	137	206	Infection persisted
Group infected with trophozoites from Monkey 2090										
2096	0 6	247	802	1077	630	156	191	286	244	Infection persisted
2102	0 6	223	690	642	880	—	—	—	—	Died from malaria
2099	40	466	616	876	410	110	368	274	199	Infection persisted
2105	40	414	394	552	738	322	152	248	—	Died from malaria

In the experiment with trophozoite induced infections, 18 monkeys were inoculated with trophozoites of the resistant strain derived from Monkey 1827 The early primary attacks, which developed in these animals, were treated with chlorguanide at daily doses of 40 mgm per kgm for a 7 day period so as to establish conclusively that each infection was resistant to therapy with this drug Having demonstrated this, treatment with either chloroquine or quinacrine was instituted 3 to 5 days after the termination of chlorguanide therapy Both drugs were administered for periods of 7 consecutive days, chloroquine at a daily dose of 2 5 mgm per kgm, quinacrine at a dose of 10 mgm per kgm Previous experiments with the parent strain of *P cynomolgi* have demonstrated that such doses of chloroquine and quinacrine are nearly always curative when employed against either early or late primary attacks or first relapses The results of the present study have been summarized in table 5, along with pertinent data on the activities of chloroquine and quinacrine against infections with the parent strain

Examination of the data in table 5 shows that chloroquine in 2.5 mgm. doses or quinacrine in 10 mgm. doses cured a high proportion of the infections with the resistant strain. The activities of these drugs against infections with this strain appeared to be at least as great as their activities against the parent strain.

The experiment with sporozoite-induced infections was limited to work with Monkeys 2089 and 2090 whose antecedents were described in section C of this report and outlined in figure 4. The primary attacks in these animals, like those in the trophozoite-induced infections described above, were first treated with chlorguanide at doses of 40 mgm. per kgm. When it was demonstrated that the infections were indifferent to chlorguanide therapy, treatment with pentaquine was instituted. The latter drug at single daily doses of 6 mgm. per kgm. was administered for 7 days. In sporozoite-induced infections with the parent strain

TABLE 5

Comparison of the activities of chloroquine and quinacrine against infections with trophozoites of the chlorguanide resistant and parent strain

DRUG	DAILY DOSE OF DRUG MGM. BASE/KGM. BODY WEIGHT	NO. OF MONKEYS TREATED	DAYS REQUIRED TO ELIMINATE PARASITEMIA	ULTIMATE RESULT	
				No. of cures	No. of relapses
Infections with chlorguanide resistant strain					
Chloroquine	2.5	7	2–3	7	0
Quinacrine	10	11	2–7	9	2
Infections with parent strain					
Chloroquine	0.6	3	3–4	0	3
	1.25	15	2–4	8	7
	2.5	17	2–3	17	0
Quinacrine	5	9	4–7	0	9
	10	14	2–6	13	1

such a dosage effects approximately 60 per cent cures. The infections in both Monkeys 2089 and 2090 were cured by this treatment.

It seems reasonable to conclude, from the data presented above, that the development of chlorguanide resistance in *P. cynomolgi* does not affect the response of trophozoite-induced infections to chloroquine or quinacrine nor the susceptibility of sporozoite-induced infections to the 8-aminoquinoline, pentaquine. As organized, the data permit no conclusions on the possibility that acquisition of chlorguanide resistance increases susceptibility to these other drugs.

Discussion. Although the development of drug resistance has been a considerable problem in some fields of chemotherapy, for example in the sulfonamide treatment of certain bacterial diseases, it has hitherto been of little importance in malaria. Different strains and different species of *Plasmodia* which infect man and lower animals exhibit significant differences in their responses to various antimalarial drugs. These, however, appear to be naturally occurring differences

and are stable characteristics of the particular strain or species. Nowhere in the voluminous literature on the clinical use of quinine is there well documented evidence showing either that the effectiveness of this alkaloid has diminished through its century long use as a suppressive or that resistance to this drug has developed during the course of treatment.

Vigorous attempts to induce quinine resistance during treatment of experimental avian infections have yielded either negative results (6) or at best only a slight change in the response of the parasite to the drug (7–9). Systematic attempts to produce quinacrine resistance have been unsuccessful (6). In the case of pamaquine some success has been obtained, several investigators (10–12) having reported the development of a moderate degree of resistance to this drug during treatment of *P. knowlesi* infections in the simian host. It is noteworthy, however, that there was no more than a fourfold change in the response of the parasite to pamaquine in these studies. In our own work with *P. cynomolgi*, of some two years duration, no evidence has been obtained for the development of resistance to either quinine or quinacrine, or to the 4-aminoquinolines, chloroquine and oxychloroquine, or to the 8-aminoquinolines, pamaquine, pentaquine, and isopentaquine.

The situation with respect to development of chlorguanide resistance is strikingly different from that just described. Less than three years experience with this drug has shown that, at least under laboratory conditions, a high degree of resistance can be produced regularly and with considerable facility. This has been demonstrated by the studies of Bishop and Birkett (1), Williamson and colleagues (2, 6), and Knoppers (9) in infections with *P. gallinaceum*, as well as by the present studies and similar work by Hawking (13) on infections with *P. cynomolgi*. There can be no question in these studies of the significance of the change in response of the parasite. Thus the change in susceptibility in the case of the avian infection was at least fortyfold, whereas that in the simian infection was more than two thousandfold.

One of the most interesting features of the work on chlorguanide resistance was the finding that the resistant characteristic which had been developed during the processes of asexual development could be transferred to the sexual forms of the parasite, retained through their reproductive processes in the mosquito and passed on to the sporozoite. This observation was made twice during the present work on *P. cynomolgi* and had been reported previously in studies on *P. gallinaceum* (1, 6). It is a finding of obvious practical importance, but it is probable that its theoretical importance is even greater. Insofar as we are aware there is no other example in biology where a characteristic produced in the asexual forms of a parasite in one host can be transferred to sexual forms, carried through an intermediary host, and reestablished in the definitive host. When one considers the complexity of changes which must occur during the exoerythrocytic and erythrocytic development of the parasite in the avian and simian hosts and the development of the sporozoite in the mosquito, the retention of the resistant characteristic is truly remarkable.

The significance of the findings of the present study, insofar as the clinical use

of chlorguanide is concerned, remains to be determined. Although there is some "word of mouth" evidence that chlorguanide is not as effective against infections with *P. vivax* and *P. falciparum* as was originally supposed, there is as yet no conclusive proof that resistance to this drug has developed during treatment of human malaria. It may be that such evidence will never be forthcoming, especially in the case of *P. falciparum* infections where chlorguanide serves as a true causal prophylactic (14). It would be much safer, however, to consider the development of chlorguanide resistance as a likely possibility and take steps to use the drug so as to minimize such an event. This could be done, we believe, by using considerably larger doses of the biguanide than were originally advocated for both suppressive and therapeutic purposes, but especially for the former. Use of larger doses, such as 0.5 or 1.0 gram twice or even three times weekly, are entirely feasible from the point of view of drug toxicity, and would involve no more administrative difficulties than the use of smaller doses.

The fact that the development of resistance to chlorguanide does not alter the response of *P. cynomolgi* to such other drugs as quinacrine, chloroquine, and pentaquine has both practical and theoretical implications. In the first instance, this fact tends to ameliorate the serious effects of chlorguanide resistance should such develop in human malaria. In the second place, it indicates that the mechanisms involved in the attack of chlorguanide on the parasite are basically different from those affected by the quinoline derivatives mentioned above.

In concluding this report, attention should be called to studies carried out in this laboratory which may well explain the ease with which resistance to chlorguanide develops and the difficulty with which resistance is acquired to such drugs as quinine, quinacrine, and chloroquine. These studies carried out on infections with *P. cynomolgi* will be reported in detail elsewhere. It is sufficient to point out at this time that chlorguanide appears to possess a parasitostatic action, which affects only the mature trophozoite and early schizont stages of the asexual cycle, other stages being unaffected. The action of chlorguanide, even on the stages mentioned above, is exceedingly gentle and results in no distinct morphological changes. Quinine, quinacrine, and chloroquine, on the other hand, exhibit a parasiticidal action and affect parasites in any stage of the asexual cycle. These latter drugs induce drastic morphological changes in the parasite, distortion of the normal shape, swelling and loss of staining characteristics of the chromatin, and vacuolation and shrinkage of the parasite to unrecognizable forms. In the case of chlorguanide, large numbers of erythrocytes containing "arrested" mature trophozoites and early schizonts persist in the peripheral blood for 2 to 5 days. In contrast the cells containing parasites, which have been killed or at least irreversibly injured by quinine, quinacrine, and chloroquine, are removed from the circulating blood in a matter of hours.

It should be pointed out at this time that the situation with respect to the parasitostatic chlorguanide and the parasitocidal drugs has obvious analogies to the sulfonamide-penicillin situation insofar as the problem of drug resistance is concerned. Resistance to the sulfonamides, which are bacteriostatic agents, can be acquired readily. Resistance to the bactericidal penicillins is difficult to attain.

The sequence of events leading to the development of chlorguanide resistant parasites is not clear at present It seems likely, however, that the persistence of "arrested" parasites, at that critical state where there is normally maximal activity of the chromatin, may afford just the right opportunity for the development of resistant forms These may appear as one of many responses of the parasite to exposure to toxic but sublethal concentrations of chlorguanide The situation may be similar to that which occurs when various plant and animal organisms are exposed to injurious but sublethal amounts of physical and chemical agents In these latter cases, mutants are said to be formed Although the suggestion may meet with considerable theoretical objection, it seems likely to us that chlorguanide may also induce mutations, one of which results in the production of chlorguanide resistant forms

SUMMARY

Preliminary observations suggested that resistance to chlorguanide could develop when infections with the trophozoites of *Plasmodium cynomolgi* were treated with subcurative doses of the above drug The present report deals with systematic investigations of this possibility and closely related problems

Experiments have shown that a high degree of chlorguanide resistance was developed when infections with *Plasmodium cynomolgi* were treated with successively increasing doses of the biguanide Such a procedure led to at least a two thousandfold change in the response of infections to the drug Thus, in monkeys inoculated with the parent strain, chlorguanide in daily doses of 0 0188 mgm per kgm regularly controlled the infection In contrast to this, in monkeys infected with the resulting resistant strain, daily administration of 40 mgm per kgm of chlorguanide, the maximum tolerated dose, was without effect on the course of the infection This resistant characteristic seems to be a well established property of the parasite, since it persisted for a period of at least 5 months during which time trophozoites were passed through 6 untreated monkeys

Transfer of the resistant characteristic through the sexual and extrinsic cycles of the parasite was also studied The result showed that the characteristic of chlorguanide resistance, which had been developed during asexual activity, was also a property of the sexual forms of the parasite and could be transmitted without loss through the mosquito

Studies were also made of the response of infections produced by the chlorguanide resistant strain to treatment with other antimalarial drugs This work showed that infections induced by trophozoites of the resistant and parent strains were equally susceptible to treatment with such drugs as chloroquine and quinacrine Likewise, infections with sporozoites of the resistant and parent strains were equally susceptible to treatment with pentaquine

The practical and theoretical significance of these findings is discussed

REFERENCES

1 Bishop, A , and Birkett, B Nature, 159 884, 1947
2 Williamson, J , Bertram, D S , and Lourie, E M Nature, 159 885, 1947
3 Porter, R Personal communication

4. MULLIGAN, H. W.: Arch. Protistenk, **84**: 285, 1935.
5. SCHMIDT, L. H., HUGHES, H. B., AND SMITH, C. C.: This Journal, **90**: 233, 1947.
6. WILLIAMSON, J., AND LOURIE, E. M.: Ann. Trop. Med., **41**: 278, 1947.
7. KRITSCHEWSKI, I. L., AND RUBINSTEIN, P. L.: Ztschr. f. Immunitätsforsch. u. exper. Therap., **76**: 506, 1932.
8. KRITSCHEWSKI, I. L., AND HALPERIN, E. P.: Ztschr. f. Immunitätsforsch. u. exper. Therap., **79**: 149, 1933.
9. KNOPPERS, A. TH.: Nature, **160**: 606, 1947.
10. NAUCK, E. G.: Arch. f. Schiffs-u. Tropen. Hyg., **38**: 313, 1934.
11. FULTON, J. D., AND YORKE, W.: Ann. Trop. Med., **35**: 233, 1941.
12. FULTON, J. D., AND YORKE, W.: Ann. Trop. Med., **37**: 41, 1943.
13. HAWKING, F., and Perry, W. L. M.: Lancet, *II*: 850, 1948.
14. FAIRLEY, N. H.: Tr. Roy. Soc. Trop. Med. and Hyg., **40**: 105, 1946.

THE *IN VITRO* PROTECTION OF EPINEPHRINE BY FLAVONOIDS

ROBERT H WILSON AND FLOYD DEEDS

Pharmacology Division, Bureau of Agricultural and Industrial Chemistry, Agricultural Research Administration, U S Department of Agriculture, Albany 6, California

Received for publication November 17, 1948

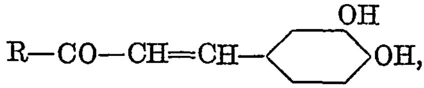
It was pointed out by one of us (F D) at the September, 1947 meeting of the American Chemical Society that the propriety of the expression "vitamin P" is debatable Vitamin P is not an entity in the sense that thiamine, riboflavin, and nicotinic acid are Such diverse substances as rutin, quercetin, epicatechin, some factors in lemon peel infusions, and even the hormone, epinephrine, possess vitamin P properties, and there is reason for believing that vitamins C, E, and K have at least one of the properties of vitamin P Moreover, no convincing evidence exists that a vitamin P deficiency ever exists in man or that it can be induced experimentally in animals except under conditions of an inadequate intake of vitamin C At a recent symposium[1] on vitamin P it was suggested, in view of the above, that the generic term "Flavonoids" be used to refer to the flavonols, flavanones and related compounds, some of which possess the pharmacological properties characteristic of vitamin P The term, flavonoids, has been used in this paper in conformity with the above suggestion

In 1941 a group of French investigators began reporting studies on the pharmacological properties of flavonoid compounds, the substances indicated by Rusznyák and Szent Gyorgyi (1) as being adjuncts to ascorbic acid in the prevention and cure of scurvy in guinea pigs These substances had few easily demonstrable pharmacological properties One of the few was the prolongation of the action of epinephrine on excised organs (Lavollay, 2) For reproducibility of results, strips of guinea pig colon were very satisfactory The increased time of action of the epinephrine was found to be due to a slower destruction, the flavonols, having antioxidant properties, slowed down oxidation of epinephrine, and as long as any of the latter was present the strips responded with characteristic relaxation (3) That the decreased destruction of epinephrine is due to a slower oxidation has been confirmed by use of the Warburg technic both by Lavollay (4) and in this laboratory (unpublished)

Lavollay (2) has reported that flavonoids did not all have the same ability to protect epinephrine He listed seven compounds (flavones, flavanones and flavonols and some of their glycosides) and indicated that, weight for weight, quercetin was more active than luteolin or rutin, which in turn were more active than morin, quercitrin, hesperetin or naringin In this paper he did not discuss the degree of activity as influenced by chemical structure, although, in a more

[1] Held under the auspices of the American Chemical Society, April 22, 1948, in Chicago, Illinois

recent publication (5) he discussed *in vivo* activity (capillary fragility measurements) in relation to structure. In the latter paper it was indicated that a number of substances with no chemical similarity had an *in vivo* vitamin P activity and that all of them could protect epinephrine from oxidation. Clark and Geissman (6) have reported briefly that for high activity the structure should be



and the o-hydroxy groups must be free.

We were fortunate in having available a number of carefully purified, naturally occurring flavonoids, and we have used these along with some chemical modifications of hesperidin, and three synthetic compounds, to study the degree of epinephrine protection and attempt to find if there was a correlation between structure and activity.[2]

METHODS. Strips of guinea pig colon were used throughout this investigation, suspended in aerated Tyrode solution in a 50-cc. bath at 38°C. Epinephrine hydrochloride was added to the bath in such amount as to cause clear but not maximal relaxation. Usually 10 micrograms was a satisfactory amount. A test substance followed by epinephrine was then added and, after recovery and washing, epinephrine alone was tried again. This was continued until the strip showed signs of fatigue or injury. The tracing obtained from test substance plus epinephrine was compared with an average of the immediately preceding and following tracings of epinephrine alone.

The exact time when a tissue is fully recovered is frequently difficult to determine. Lavollay (2) suggested that for comparing one substance with another, the "half-recovery" time be used. This is the time required for the muscle to relax and return half-way to its original length; at this point the slope of the curve is steep enough to make measurement easy. All of our measurements were of half-recovery time.

The flavonoids have a low water solubility. In most instances solubility was possible, with or without warming, in 5 per cent ethyl alcohol in Tyrode solution. In some cases, such as hesperidin, propylene glycol was necessary in concentrations up to 50 per cent. None of these solvents in amounts up to 1 cc. in the 50-cc. bath affected the response of the tissue to epinephrine. Since the usual addition was 0.5 cc. or less, effects of the solvents could be ignored. All flavonoid solutions were freshly prepared just before use, since we noticed early in the experiment that rutin solutions, which in some instances had been preserved in a refrigerator for several days, degenerated on standing.

Comparisons were made on the basis of molecular equivalents rather than equal weights. The standard of reference was quercetin, 0.128 mgm. of this compound per 50 cc. of bath solution being used in all instances. This amount was equivalent to 0.25 mgm. of rutin, a

[2] We want to thank the Eastern Regional Research Laboratory, Bureau of Agricultural and Industrial Chemistry, U.S.D.A., for the rutin, quercitrin and quercetin (7); the California Fruit Growers' Exchange for hesperidin and the methylated hesperidin chalcone; the Ingram Laboratories, Inc., San Francisco, for the hesperidin chalcone and its sodium salt; Dr. W. G. Clark for the methylated chalcone; the Forest Products Laboratory, Forest Service, U.S.D.A., for the dihydroquercetin and dihydrokaempherol (8); S. B. Penick and Co. for the xanthorhamnin; and the Enzyme Research Laboratory, Bureau of Agricultural and Industrial Chemistry for the *o*-hydroxychalcone. Also the following flavonoid compounds were studied: isoquercitrin (9), quercetin galactoside (10), quercimeritrin (11), kaempherol and robinin (12, 13), and genistin (14).

quantity of rutin which caused epinephrine to relax the muscle for a time definitely longer than normal, but without unduly prolonging the time. Each strip was tested with this amount of quercetin and with molar-equivalent amounts of other substances. The *increase* in half-recovery time produced by quercetin was given the value of 1.00. Repeated tests with quercetin showed that most strips degenerated slowly as shown by decreasing values of half-recovery time. This has been compensated for, as much as possible, by introducing the quercetin more than once for each strip, by introducing it in the middle of each experiment, or, knowing the usual rate of deterioration of the strips, by calculation. When the test substance had no effect on the half-recovery time, three or four tracings with different strips on different days were considered sufficient. When there was activity, four to ten or more tracings were made, the greater number being for those substances where variation was most marked. The values reported in the table are means of the individual values and are probably within 20 or 25 per cent of the true means, as judged by repeated assays of quercetin.

In our earlier report (15) on the pharmacology of rutin we stated that we had verified the in vitro protection of epinephrine by rutin and quercetin. It was further stated that if the concentrations of these flavonols were great enough, there was a direct relaxing effect on the intestinal strips. In the work reported in the present paper, the concentrations of the various flavonols tested were such that in no case was a direct effect noted.

Since some of the assays were done with aeration tubes made of copper, and since copper is apt to catalyze oxidations, some experiments were made in an all-glass apparatus, with and without added copper. The epinephrine half-recovery times were decreased in the presence of copper, but the increased half-recovery times, on a percentage basis (the basis for all comparisons in this paper) were not affected.

Results. The results are presented in table I. It is apparent that the presence of a monosaccharose or, in the case of rutin, a disaccharose in the 3-position does not greatly modify the activity of quercetin. On the other hand, substitution of a single sugar, glucose, in the 7-position increased the activity to a very considerable extent. When the 7-position is covered by methoxylation, as in xanthorhamnin, activity seems to be reduced. This statement cannot be made without reservation, however, since in addition to the methylation, there is a trisaccharose on C-3. There is no reason to suppose that a trisaccharose would modify activity when a disaccharose (as in rutin) does not, but this difference must be considered.

Hesperetin, a flavanone, differs from quercetin by a reduction which eliminates the double bond and by methylation of one of the phenyl hydroxyls. Its glycoside, hesperidin, has no ability to prolong the action of epinephrine on the intestinal strip. When the central ring (pyrose nucleus) is opened to form hesperidin chalcone or its methylated derivatives, a double bond is again formed but no activity appears. The simpler compound, chalcone (benzalacetophenone), has some activity, and the *o*-hydroxy derivative of chalcone also has a slight action. Methoxylation on the phenyl ring decreases the epinephrine protecting action of chalcone to a very considerable extent.

Kaempherol differs from quercetin in having a single hydroxyl on the phenyl ring. It has some activity, which is decreased but not completely eliminated when saccharoses are placed in the 3- and 7-positions (robinin). Hydrogenation of quercetin decreases, but does not eliminate, activity while hydrogenation of kaempherol causes complete disappearance of epinephrine protecting action.

TABLE I

The protection of epinephrine from oxidation as indicated by intestinal strips

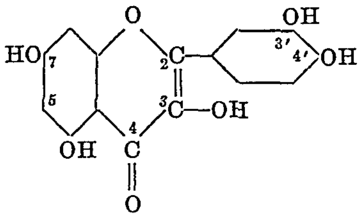
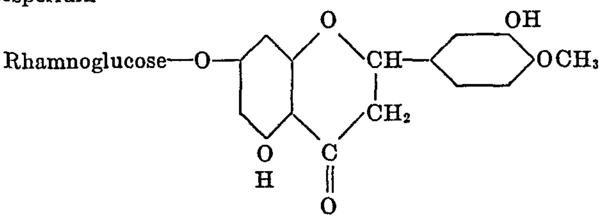
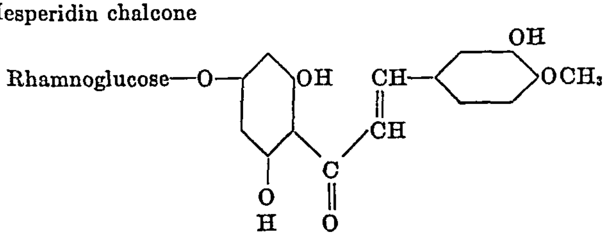
MATERIAL	MOLAR EQUIVALENTS	INCREASE IN EPINEPHRINE HALF-RECOVERY TIME
Quercetin and derivatives	1	1.0 (by definition)
		
Substitution on C-3		
Monosaccharose		
Quercitrin (rhamnose)	1	1.1
Isoquercitrin (glucose)	1	1.2
Quercetin galactoside	1	1.3
Disaccharose		
Rutin (rhamnoglucose, rutinose)	1	1.0
Substitution on C-7		
Monosaccharose		
Quercimeritrin (glucose)	1	2.0
Substitition on C-3 and C-7		
Trisaccharose on C-3, methoxy on C-7		
Xanthorhamnin	1	0.6
Flavanones and derivatives		
Hesperidin	4	0.1
		
Hesperidin chalcone	4	0.1
		

TABLE I—*Continued*

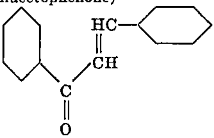
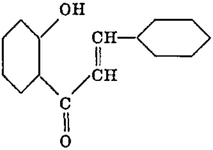
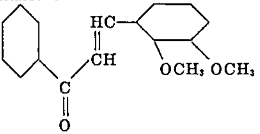
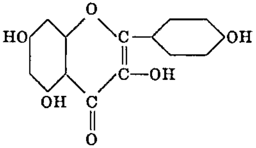
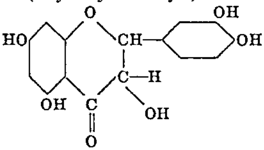
MATERIAL	MOLAR EQUIVALENTS	INCREASE IN EPINEPHRINE HALF RECOVERY TIME
Sodium hesperidin chalcone	4	0
Methylated hesperidin chalcone	4	0
Chalcone (benzalacetophenone)	1	0
	6	0 9
o Hydroxychalcone	1	0
	4	erratic (0 to ∞)
2′,3′ Methoxychalcone	4	0
	12	∞
Similar structures		
Kaempherol	1	0 1
	2	0 4
	4	1 0
Robinin	4	0 1
(A 3 rhamnogalactoside, 7 rhamnoside of kaempherol)	8	0 4
Dihydroquercetin (3 hydroxy eriodictyol)	0 9	0 3
	3	0 8

TABLE I—*Continued*

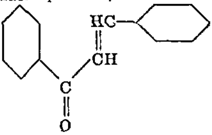
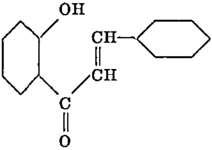
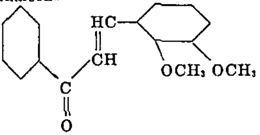
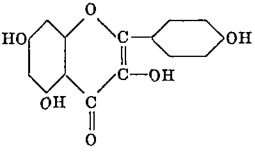
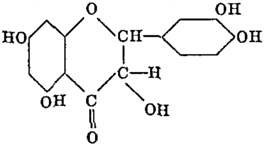
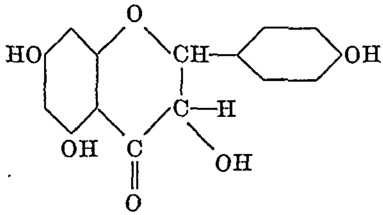
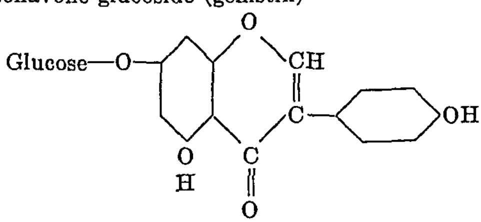
MATERIAL	MOLAR EQUIVALENTS	INCREASE IN EPINEPHRINE HALF RECOVERY TIME
Sodium hesperidin chalcone	4	0
Methylated hesperidin chalcone	4	0
Chalcone (benzalacetophenone)	1	0
	6	0 9
o-Hydroxychalcone	1	0
	4	erratic (0 to ∞)
2′,3′ Methoxychalcone	4	0
	12	∞
Similar structures		
Kaempherol	1	0 1
	2	0 4
	4	1 0
Robinin	4	0 1
(A 3 rhamnogalactoside, 7 rhamnoside of kaempherol)	8	0 4
Dihydroquercetin (3 hydroxy eriodictyol)	0 9	0 3
	3	0 8

TABLE I—*Concluded*

MATERIAL	MOLAR EQUIVA-LENTS	INCREASE IN EPINEPHRINE HALF-RECOVERY TIME
Dihydrokaempherol (3-hydroxynaringenin) 	14	0
Isoflavone glucoside (genistin) 	3	0

These two hydrogenated compounds are more closely related to hesperetin than to quercetin and kaempherol, being flavanones rather than flavonols.

Isoflavone glucoside (genistin) is a compound chemically resembling the very active quercimeritrin in that it has a glucose in the 7-position, but it differs in that the phenyl grouping has been changed from the second to the third carbon and the hydroxyl on the carbon 3 has disappeared. These changes result in complete loss of activity.

Discussion. The importance of certain chemical configurations can be judged to a degree by the data just presented. There are gaps in the picture so that none of the conclusions can be considered as final. None the less, some tentative conclusions are justified.

A glycosidal linkage on C-3 has little or no effect on the ability of quercetin to prolong the action of epinephrine on an intestinal strip. In the C-7 position, however, such a linkage augments the activity considerably. The importance of the C-7 position is emphasized by the loss of activity occurring when that hydroxyl is methylated. Robinin was the only compound studied in which saccharoses were present in more than one position. It would be unwise, on the basis of this one example, to draw any conclusions as to why such substitution decreased activity.

Hesperidin has a glycosidal linkage on C-7, but it differs from the very active quercimeritrin by a loss of the double bond between C-2 and C-3, and by the methylation of one of the phenyl hydroxyls. It has lost all activity. If the double bond was the important factor in the activity, then the chalcone of hes-

peridin should protect epinephrine, which it does not Therefore, it would seem that two free hydroxyls ortho to each other on the phenyl ring might be necessary Two such hydroxyls are important, but they are not the complete answer, since kaempherol, with one hydroxyl has some activity, though much less than quercetin, which has two

Apparently both the double bond between C 2 and C-3 (or the corresponding carbons when the ring has been opened) and an *o* dihydroxyphenyl group are important The importance of the double bond is shown by the activity of chalcone and its hydroxy derivative The activity is not great, but it is present Again, loss of the double bond by reduction of quercetin to dihydroquercetin causes decrease in activity, as does the corresponding reduction of kaempherol In the latter case, the decrease is to complete loss of activity

In one compound (chalcone) no hydroxyls were on the phenyl ring and yet there was a certain protection of epinephrine However, hydroxyls are important, and two are better than one Quercetin, with two, has more activity than kaempherol, with one hydroxy group on this ring, and dihydroquercetin is better than dihydrokaempherol So, while activity has been demonstrated in compounds lacking either the double bond or the dihydroxyphenyl group, our results are in accord with those of Clark and Geissman that for marked activity both of these structures are necessary

Judging by the one example, the phenyl group should be in position 2 In the isoflavone glucoside, the glucose is in the very favorable C 7 position, and yet there is complete loss of activity The loss of activity is probably not due to the lack of a hydroxyl group on C 2, since the covering of the C-3 hydroxyl of quercetin by glycosidal linkage did not modify activity The loss of activity is presumably due to the movement of the phenyl group to the 3 position

It must be remembered that these *in vitro* results are not necessarily the same as those in an intact animal Oxidations and reductions, often difficult in the laboratory, may be accomplished easily and very quickly in the animal body

SUMMARY AND CONCLUSIONS

A number of flavonols, flavanones, certain of their derivatives, some of which are considered to have vitamin P activity, and some synthesized, related compounds, have been tested for their ability to protect epinephrine from destruction, using an isolated strip of intestine as the test object It is suggested that, on an equimolecular basis, the following tentative conclusions may be justified

1 A glycosidal linkage on the C-3 position does not modify activity

2 A glycosidal linkage on C-7 increases, while methoxylation of this group decreases, activity

3 A double bond between C 2 and C 3 increases activity

4 Two free hydroxyl groups, ortho to each other, on the phenyl ring also increase activity

ACKNOWLEDGMENT Dr C E Sando has aided us immeasurably with his knowledge of the chemistry of the flavonoids and his enthusiastic interest in the progress of this work

REFERENCES

1. Rusznyák, St., and Szent-Györgyi, A.: Nature, **138**: 27, 1936.
2. Lavollay, J.: Compt. rend. soc. de Biol., **135**: 1193, 1941.
3. Lavollay, J.: Compt. rend. acad. Sci., **214**: 287, 1942.
4. Lavollay, J.: L'autoxydation des diphénols en particulier de l'adrénaline. Structure et rôle fonctionnel de la vitamine P. Hermann et cie., Paris, 1942.
5. Javillier, M., and Lavollay, J.: Helv. Chim. Acta, **29**: 1283, 1946.
6. Clark, W. G., and Geissman, T. A.: Fed. Proc., **7**: 21, 1948.
7. Couch, J. F., Naghski, J., and Krewson, C. F.: Science, **103**: 197, 1946.
8. Pew, J. C.: J. Am. Chem. Soc., **70**: 3031, 1948.
9. Sando, C. E., and Bartlett, H. H.: J. Biol. Chem., **54**: 629, 1922.
10. Sando, C. E.: J. Biol. Chem., **117**: 45, 1937.
11. Sando, C. E.: J. Biol. Chem., **68**: 407, 1926.
12. Sando, C. E.: J. Biol. Chem., **94**: 675, 1931–32.
13. Zemplén, G., and Bognár, R.: Ber. deut. chem. Gesellschaft, **74**: 1783, 1941.
14. Walter, E. D.: J. Am. Chem. Soc., **63**: 3273, 1941.
15. Wilson, R. H., Mortarotti, T. G., and DeEds, F.: This Journal, **90**: 120, 1947.

CENTRAL IMPAIRMENT OF SYMPATHETIC REFLEXES BY 8 AMINOQUINOLINES[1]

GORDON K MOE, BRAULIO PERALTA,[2] AND MAURICE H SEEVERS

Department of Pharmacology, University of Michigan, Ann Arbor

Received for publication November 24, 1948

In the course of routine analysis of the acute and subacute toxicity of a number of antimalarial compounds during the war, observations of the toxic effects of quinacrine and of pamaquine on the dog were made for comparative purposes It was found that pamaquine salts administered orally in divided doses equivalent to 5 mgm of base per kilogram per day for 3 to 6 days caused ocular changes suggestive of sympathetic paralysis enophthalmos, pupillary constriction, and relaxation of the nictitating membranes A survey of early pharmacological studies of pamaquine failed to reveal any similar previous observations, most of these papers dealt primarily with the production of methemoglobinemia in various species The present study was begun in an attempt to elucidate the mechanism of the apparent paralysis of the sympathetic supply to the eye

Preliminary results demonstrated that in addition to impairment of ocular mechanisms, sympathetic cardiovascular reflexes were also depressed or abolished by pamaquine (1) Schmidt *et al* (2) described among other signs of pamaquine intoxication in the dog, divergent strabismus, relaxation of the nictitating membrane, and loss of pupillary reflexes, plasmocid was reported to produce central nervous system damage, particularly in the monkey, and it was thought possible that the effects of pamaquine observed by us might be due to actual destruction of central cell stations.

When it was learned that Alving and his co-workers had observed postural hypotension in human subjects receiving high doses of pentaquine (observations recently reported, 3), our study was extended to include pentaquine and other compounds closely related to pamaquine[3] Preliminary studies with pentaquine by Richardson, Walker, and Miller (4) yielded results which are fully supported by the data reported below

The compounds used, derivatives of 6 methoxy-8 aminoquinoline, are listed below

Pamaquine (SN-971) 8-(4 diethylamino 1-methylbutylamino) 6 methoxyquinoline

Pentaquine (SN-13,276) 8-(5 isopropylaminoamylamino) 6 methoxyquinoline

[1] Supported by grants from the Office of Scientific Research and Development and from the Research Grants Division of the United States Public Health Service

[2] Rockefeller Fellow Present address, E R Squibb and Sons, Mexico, D F

[3] Pamaquine was obtained as the hydroiodide, naphthoate, and citrate from the Survey of Antimalarial Drugs Pentaquine was obtained as the phosphate from the Survey Office and from the National Institute of Health SN 13 274 was obtained from the Abbott Laboratories and SN 13,140 from the Survey Office

Isopentaquine (SN-13,274): 8-(4-isopropylamino-1-methylbutylamino)-6-methoxyquinoline.

SN-13,140: *8-[-2-(2-piperidyl)-isopropylamino]-6-methoxyquinoline.*

METHODS. Dogs and rhesus monkeys were used. Cats were found unsuitable because of the development of severe methemoglobinemia. The drugs were administered orally twice a day in gelatin capsules or injected intramuscularly 2 or 3 times daily in aqueous solution. Dogs were fed a stock diet of Purina Dog Chow, supplemented on occasion with raw horse meat or canned dog food. Monkeys were supplied with Purina Dog Chow, wheat, tomato juice and apples.

After 3 to 10 days of treatment, terminal acute experiments were performed under thiopental-barbital, pentobarbital, or chloralose anesthesia. Great caution was necessary in judging the anesthetic dose for severely intoxicated animals, which were often adequately depressed with half the ordinary dose. Blood pressure, respiration and heart rate were recorded by the usual methods. Methemoglobin estimations were carried out occasionally during the period of treatment to rule out the possibility of "chronic" anoxia as the cause of the changes observed. In none of the animals considered below were methemoglobin levels higher than 15 per cent of the total hemoglobin; anoxia could hardly have been responsible for the changes observed.

Acute experiments were carried out on 6 dogs and 3 monkeys treated chronically with pamaquine, 6 dogs and 4 monkeys treated with pentaquine, 3 dogs and 2 monkeys treated with isopentaquine, and 2 dogs treated with SN-13,140. In the subsequent discussion, "acute" experiments refer to such terminal studies of animals intoxicated by repeated daily administration of the various drugs.

RESULTS. *Dog:* On the daily dosage of 5 mgm. base/kgm. of pamaquine all animals lost weight rapidly. Food and water intake diminished, and eventually even fresh horse meat was refused. Spontaneous activity diminished. If carried too far, the animals would die in spite of withdrawal of the drug and treatment with intravenous glucose and saline. Although injection of ephedrine improved the outward appearance of severely depressed animals and temporarily restored appetite, no increase in survival time could be demonstrated when this agent was administered routinely during the course of treatment. Some animals developed purulent infections of the conjunctivae, nasal and urethral mucosae, or of slight cutaneous abrasions. Such animals were not used for the terminal acute experiments. Pentaquine, 10 mgm. base/kgm./day, and isopentaquine, 7.5 to 10 mgm./kgm./day, were better tolerated although they produced more nearly complete suppression of sympathetic reflexes.

By the third to sixth day of treatment with these agents the characteristic eye signs appeared; when these had become marked and while the dogs were still in relatively good condition, they were anesthetized and subjected to various acute tests of autonomic function, including (a) carotid occlusion, to test the integrity of pressor reflexes; (b) centripetal stimulation of the cervical vagosympathetic trunk, to test the function of the peripheral sympathetic neurohumoral mechanism, and to provoke reflex responses of heart rate and blood pressure; (c) centrifugal stimulation of the vagi; (d) injection of epinephrine, to test the responsiveness of arteriolar smooth muscle as well as the reflex carotid sinus cardio-decelerator and respiratory-inhibiting mechanisms; (e) injection of nicotine to test the carotid body respiratory stimulant reflex and the integrity

of sympathetic ganglia, (f) injection of tetraethylammonium to estimate the levels of sympathetic tone to heart and vessels, (g) injection of veratridine, to test for reflex bradycardia, apnea, and hypotension, (h) stimulation of the carotid sinus nerve, and (i) asphyxia In general, the responses showed a nearly complete loss of reflex sympathetic control over heart and circulation, with preservation of parasympathetic cardiovascular reflexes and of respiratory responses, results which conform with those reported for pentaquine by Richardson *et al* (4)

The blood pressure and heart rate of all treated dogs under barbiturate anesthesia averaged 88 mm Hg and 105 beats/min as compared with 151 and 180 in 9 control animals These levels are compatible with nearly complete suppression of sympathetic tone to heart and vessels In all animals treated with pamaquine for a sufficiently long period, carotid occlusion caused no elevation of heart rate, and a much smaller elevation of pressure than in control animals (table 1, figure 1A) The figures in the table are percentage responses, the *absolute* change of pressure induced in treated animals by carotid occlusion was very much less than in control animals Pentaquine completely abolished the effects of carotid occlusion in 4 of the 6 dogs Removal of the aortic buffer influences by vagal section in control animals raised the arterial pressure and enhanced the response to carotid clamping, but this procedure was without effect in the treated dogs Centripetal vagal stimulation, which in control animals caused cardioacceleration and hypertension, failed to affect the pressure or heart rate in treated animals, except occasionally as a result of apnea, hyperpnea, or gross muscle movements (figure 1A) The alterations of these responses could result from functional impairment either centrally or along the efferent

TABLE 1

Responses of pamaquine dogs compared with control animals

ANIMAL	DOSE	DAYS TREATED	B P RESPONSES, PER CENT			HEART RATE RESPONSES, PER CENT		
			Carotid clamp	T E A	*Asphyxia*	Carotid clamp	T E A	*Asphyxia*
	mgm /kgm / day							
173	5	3½	+15	−42	0	0	−6	0
244	5	6	+19	−40	0	0	−3	0
258	5	3½	+7	−43	0	0	−12	0
260	3 5	3½*	0	−18	0	0	−6	0
271	5 5	6	+3	−9	0	0	0	0
259†	3 0	10	+25	−38	+20	+11	−14	+13
Average‡			+9	−30	—	—	−5	—
Average 9 controls			+30	−40	+36	+12	−23	+19

* Treated for 18 days previously with smaller doses

† No ocular signs present at time of acute experiment

‡ Excluding dog #259

pathways involved; however, centripetal stimulation of the vagosympathetic trunk caused protrusion of the globe, retraction of the nictitating membrane, and dilatation of the pupil, illustrating the integrity of the ganglionic and terminal neurohumoral mechanisms. Centrifugal vagal stimulation caused heart block or cardiac arrest, demonstrating the integrity of peripheral parasympathetic mechanisms.

The low level of pressure might have been the result of shock; in that case, the pressor effect of epinephrine would have been reduced. This was not the case. Epinephrine caused a greater and more prolonged elevation of pressure than in control animals (figure 1A). Respiration was slowed or briefly arrested at the peak of the pressor response, proving the integrity of the pressoreceptors and their reflex connections through the respiratory centers. The heart rate

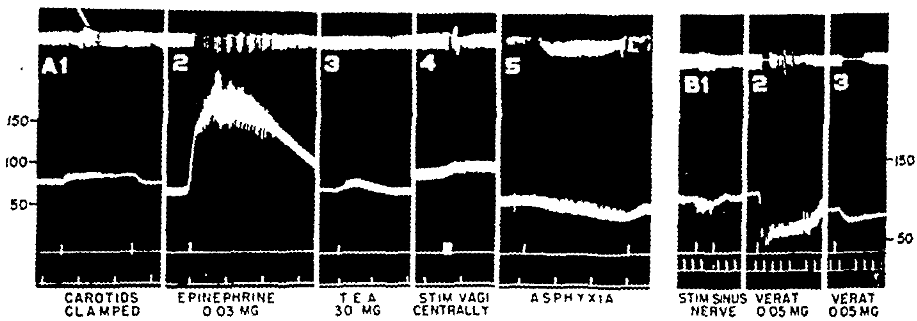


FIG. 1A. DOG, 10 KGM., PENTOBARBITAL ANESTHESIA

Treated for 4 days with pentaquine, 10 mgm./kgm./day. Tracings from top to bottom: respiration, arterial pressure (scale at left), signal, time in minutes. Between segments 3 and 4, a lapse of time sufficient for complete recovery from tetraethylammonium (T.E.A.). *Asphyxia produced by respiration of a mixture of 90% N_2, 5% O_2, and 5% CO_2.*

FIG. 1B. DOG, 8 KGM., PENTOBARBITAL ANESTHESIA

Treated for 6 days with pamaquine, 5 mgm./kgm./day. Tracings as in 1A, but time in 10 sec. Segment 1, stimulation of Hering's nerve causes bradycardia but little depressor response. Between 2 and 3, atropine, 0.1 mgm./kgm. Verat. = veratridine.

was significantly slowed by epinephrine, even to the point of assumption of an A-V-nodal rhythm, again attesting to the integrity of the pressoreceptors and their reflex vagal connections.

The integrity of the autonomic ganglia was further proved by the response to nicotine, which caused its characteristic effects on heart rate and blood pressure as well as hyperpnea resulting from stimulation of carotid-body chemoreceptors.

The absolute depressor response to tetraethylammonium (T.E.A.) was reduced, although in most of the pamaquine-treated animals the *percentage* reduction of pressure remained normal (table 1). Since T.E.A. reduces blood pressure as the result of blockade of autonomic ganglia, it is obvious that the dogs retained a certain degree of sympathetic tone to the arterioles. Except in 2 animals pentaquine completely abolished the response to tetraethylammonium, as illustrated in segment 3 of figure 1A.

Veratridine, which causes reflex bradycardia, hypotension, and apnea as a result of stimulation of certain vagal afferents in the heart and lungs (5) pro-

duced its characteristic effects on heart rate and respiration, but when the bradycardia was prevented by atropinization, the hypotension was limited to the same slight response as with T E A (figure 1B, segments 2 and 3, before and after atropine.)

Stimulation of Hering's nerve, which in control animals caused a considerable fall of pressure, was almost without action on the blood pressure of the treated dogs, but still evoked a slowing of the heart (figure 1b, segment 1)

Asphyxia, a potent pressor stimulus in control animals, caused only a fall of pressure in the treated animals (figure 1A).

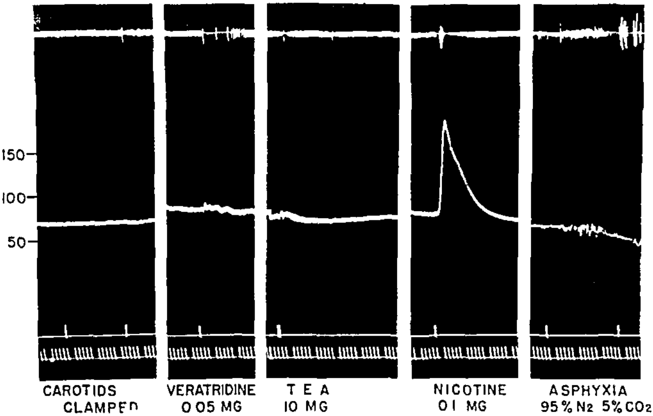


Fig 2 Monkey, 3 3 Kgm , Chloralose Anesthesia

Treated for 6 days with pentaquine, 10 mgm /kgm /day Tracings as in previous figure, time in 10 sec

The observed impairment of ocular sympathetic mechanisms appeared to be irreversible Two dogs, maintained on pamaquine until development of the characteristic eye signs, were taken off the drug and observed for 3 months At the end of that time only partial recovery of sympathetic control of the eye was apparent

Since SN-13,140 was known to produce a different pattern of toxic effects, 2 acute experiments were carried out for comparison in dogs having received this drug for 5 days in doses producing severe intoxication as indicated by anorexia, weight loss, and depression In both experiments blood pressure and heart rate were normal, and sympathetic reflexes were unaffected It was concluded that the effects observed with pamaquine, pentaquine, and isopentaquine were not due merely to debilitation, but probably represented a specific interference with autonomic functions

Monkeys Administration of pamaquine in the monkey produced extreme

anorexia and weight loss, depression, and methemoglobinemia. Two monkeys brought to acute experiment were moribund, and the failure to demonstrate vascular reflexes could not be assigned with assurance to specific brain damage induced by the drug; the third monkey, used before signs of severe intoxication developed, reacted normally to all test procedures.

Pentaquine was tolerated much better than pamaquine, and 4 animals remained in good condition after medication 3 times daily for 3 to 10 days (10 mgm. base/kgm./day). The end-point was difficult to judge because of lack of ocular signs, but in terminal acute experiments these 4 animals reacted like the dogs. Blood pressure and heart rate were lower than in control animals; pressor responses to carotid clamping, central vagal stimulation and asphyxia, and the depressor response to tetraethylammonium were lost, while the pressor and hyperpneic response to nicotine was retained (figure 2).

The effects of isopentaquine after daily dosage of 7.5 to 15 mgm. base/kgm. were essentially similar to those produced by pentaquine.

Discussion. From the available data it is concluded that these 8-aminoquinolines cause central impairment of certain sympathetic regulatory mechanisms, particularly those controlling blood pressure and heart rate. Although histological examination of the brains was not obtained, the chronic nature of the sympathetic involvement, and the demonstrated neuro-toxicity of various 8-aminoquinolines suggest that the impairment may be due to actual destruction of central neurones. In dogs treated with pamaquine, destruction could not have been complete, for a significant response to tetraethylammonium was retained. Since reflex pressor responses were nearly completely suppressed, one may conclude that what remained of vasomotor mechanisms must have been discharging maximally to maintain arterial pressure even at its reduced level, leaving no buffer reserve for further reflex elevation. Carotid and vagal afferent mechanisms were shown to be intact; furthermore, depression or destruction of such paths peripherally without additional damage elsewhere would cause hypertension, not hypotension. Efferent pathways through and beyond autonomic ganglia were shown to remain functionally intact. The lesion then must lie either in the brain or cord.

Examination of the central nervous system in dogs and monkeys chronically poisoned with pamaquine failed to reveal significant damage to sympathetic pathways in the spinal cord or to the lateral horn cells (Schmidt and Schmidt, 6). Only moderate damage was demonstrated in the medulla, mid-brain and hypothalamus. Studies of the brains of dogs treated with pentaquine and isopentaquine have not been completed (6); in the monkey these agents produce lesions in the supraoptic and paraventricular nuclei as well as in the abducens, trochlear, and oculomotor nuclei (6). Although lesions to which the sympathetic reflex impairment described above could be assigned have not been described, it is probable that the neurones involved in the central mediation of these reflexes at the medullary level are not closely grouped; damage to such diffuse "centers" might escape notice. The suppression of autonomic reflexes can not have resulted from damage to hypothalamic nuclei, for it is known that carotid sinus reflexes are accomplished at the medullary level; clamping the

carotid arteries still causes a pressor response in animals whose brain-stem has been transected below the hypothalamus. The lesion is therefore probably located in the medulla. This does not, of course, imply that nuclei contributing to autonomic regulations from a higher level are not also damaged.

Strabismus induced by the 8-aminoquinolines may be accounted for by the demonstration of lesions of the motor nuclei of the III, IV, and VI cranial nerves, although the damage to these nuclei produced by pamaquine in the dog is said not to be severe (6, 7).

Species differences in the response to chronic administration of pamaquine and its congeners are marked. The rat, for example, shows little or no evidence of brain damage (2). Pamaquine, which produces evidence of extensive impairment of sympathetic reflexes in the dog, exerts its primary toxic actions in the monkey on the hematopoietic system (2), while pentaquine causes similar impairment of sympathetic reflexes in the two species. The observations of Alving and his co-workers suggest that pentaquine produces damage to sympathetic mechanisms in the human subject also (3). Administration of the drug in maximum doses produced postural hypotension lasting for months after withdrawal of the drug. Freis and Wilkins have made a preliminary attempt to apply this toxic action in the symptomatic therapy of hypertension (8). Although arterial pressure was diminished, intolerable side effects forced cessation of the medication.

The mechanism of the damage produced by these and related compounds is unknown. Schmidt and Schmidt (7), on the basis of their careful histological studies, believe that the extensive changes produced by plasmocid are not the result of general or local circulatory disturbance, nor to hemorrhagic lesions. Hypoxia, resulting from methemoglobinemia and hypotension, could not have been severe; methemoglobin concentrations were not excessive; and, indeed, areas most sensitive to the drugs have been shown to be very resistant to anoxia or circulatory arrest (9). Severe lesions may occur in discrete neurone groups while neighboring nuclei exhibit little or no evidence of damage.

Because of the remarkable selectivity of the various 8-aminoquinolines, these agents should become useful tools in the study of autonomic as well as somatic functions.

ACKNOWLEDGMENT. The authors are indebted to Mr. Carl Gruhzit and Mr. Joseph Cochin for their aid in some of these experiments.

SUMMARY

1. Pamaquine, pentaquine, and isopentaquine chronically administered to dogs produce impairment of sympathetic cardiovascular reflexes, but not of parasympathetic or respiratory reflexes.

2. Pentaquine and isopentaquine, but probably not pamaquine, cause similar effects in the monkey.

3. The impairment of reflexes is believed to be due to destruction of medullary cell-groups.

REFERENCES

1. Moe, G. K., and Seevers, M. H.: Fed. Proc., 5: 193, 1946.
2. Schmidt, L. H., Smith, C. C., Hughes, H. B., and Carter, C.: Fed. Proc., 6: 369, 1947.
3. Craige, B., Eichelberger, L., Jones, R., Alving, A. S., Pullman, T. N., and Whorton, C. M.: J. Clin. Investigat., 27: supplement, p. 17, 1948.
4. Richardson, A. P., Walker, H. A., and Miller, B. S.: Proc. Soc. Exper. Biol. and Med., 65: 258, 1947.
5. Moe, G. K., Bassett, D. L., and Krayer, O.: This Journal, 80: 272, 1944.
6. Schmidt, I. G., and Schmidt, L. H. (Personal Communication).
7. Schmidt, I. G., and Schmidt, L. H.: Fed. Proc., 6: 368, 1947. Also, J. Neuropathol. & Exper. Neurol., 7: 368, 1948.
8. Freis, E. D., and Wilkins, R. W.: Proc. Soc. Exper. Biol. and Med., 64: 455, 1947.
9. Grenell, R. G., and Kabat, H.: J. Neuropathol. & Exper. Neurol., 6: 35, 1947.

THE ALTERED BLOOD-PRESSURE RESPONSE AFTER ADRENOLYTIC DRUGS AND LARGE DOSES OF SYMPATHOMIMETIC AMINES[1]

I. A. CORET AND H. B. VAN DYKE

Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York

Received for publication November 26, 1948

In experiments in which two types of adrenolytic drugs were used, a large intravenous dose of a sympathomimetic amine could peculiarly modify the blood pressure response to small doses of the same amine. For example, if an immense dose of epinephrine had been administered after an adrenolytic drug, the injection of a small dose of epinephrine produced no effect rather than the expected purely depressor response. When the study was extended to depressor sympathomimetic amines such as the isopropyl homologue of epinephrine, Isuprel, it was found that a large dose of this substance temporarily blocked the depressor action of small doses of Isuprel. Furthermore, the usual depressor response to epinephrine was again reversed and a pressor response was elicited. The effect of epinephrine, Isuprel or related drugs in abolishing a depressor response will be described as a "tapenolytic" effect (ταπείνωσις = process of lowering or depressing + λύειν = to dissolve)[2].

Cats anesthetized with intraperitoneal pentobarbital, Dial with urethane or intravenous α chloralose were used. Atropine sulfate in a total dose of 1–2 mgm. was administered intramuscularly. The adrenolytic (sympatholytic) drugs used were drug 194[3], Dibenamine and the methane sulfonates of dihydroergocristine and dihydroergocryptine. The adrenolytic drugs and the sympathomimetic amines were injected intravenously. Details of technique are given in a previous report (1).

Effect of massive doses of catechol amines on the block produced by adrenolytic agents. Large doses of catechol amines may drastically affect the lysis produced by a blocking agent. For example, if 2.0 mgm. per kgm. of epinephrine were injected after the maximum adrenolytic effect of 15 μM per kgm. of drug 194 had appeared, a subsequent test injection of 6 microgm. per kgm. of epinephrine produced no response. Thus, as revealed by the absence of a depressor response to the small test dose of epinephrine, the large dose of epinephrine had produced tapenolysis. Repetition of this small test dose at regular intervals later caused the gradual reappearance of a depressor response; therefore, tapenolysis was reversible and the effects of the adrenolytic drug persisted.

[1] This investigation was supported by a grant from the Smith, Kline and French Laboratories.

[2] A more correct term would be "sympathotapenolytic" since discussion is limited to the reduction or abolition of depressor sympathetic responses.

[3] Drug 194 was furnished by the Smith, Kline and French Laboratories. It is N benzyl N β phenylisopropyl β chloroethylamine hydrochloride. Pharmacologically it is similar to Dibenamine (dibenzyl β chloroethylamine hydrochloride).

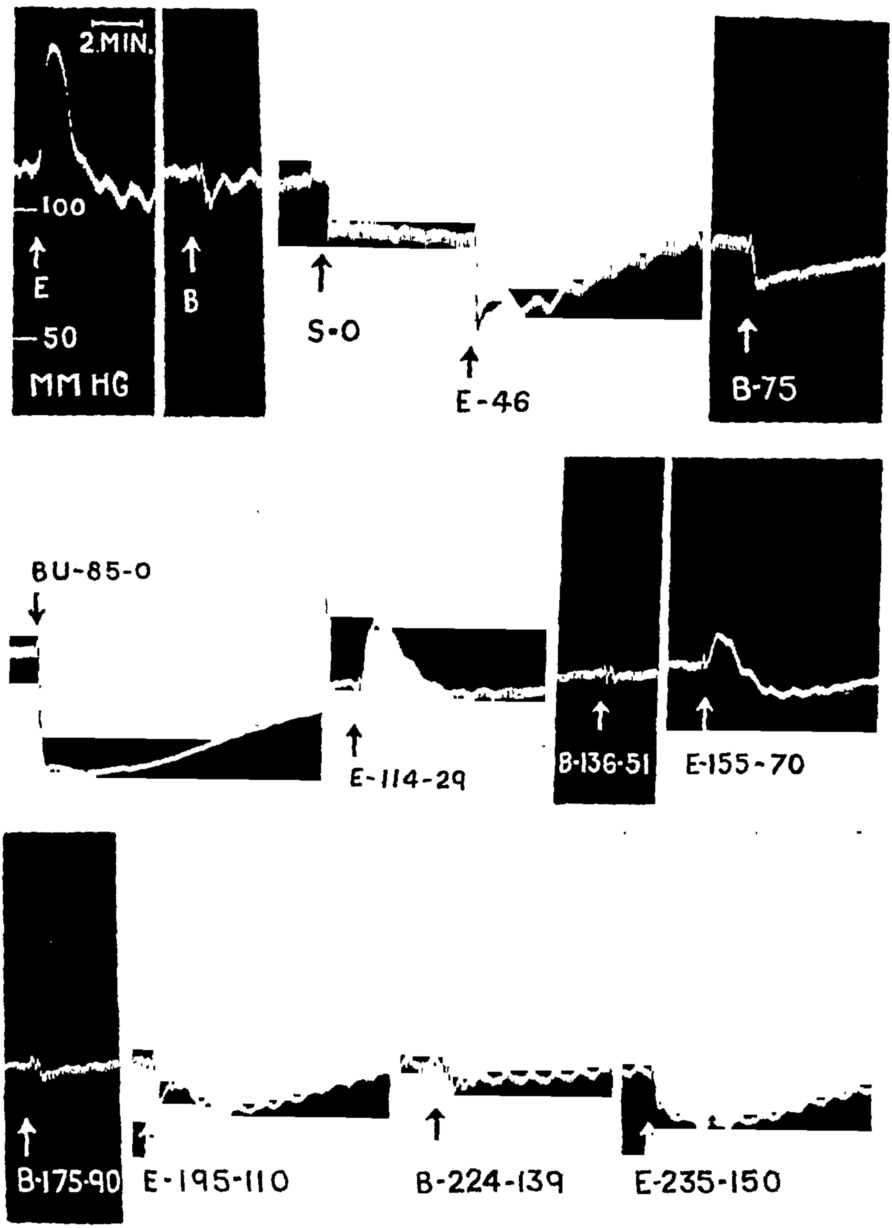


FIG. 1. TAPENOLYSIS PRODUCED BY ETHYLNOREPINEPHRINE (BUTANEPHRINE)

Blood pressure of cat, 2.6 kgm. Pentobarbital anesthesia. 2 mgm. atropine sulfate intramuscularly. E: epinephrine HCl, 6 microgm. per kgm.; B: Butanephrine HCl, 4 microgm. per kgm.; S-O: 15 μM per kgm. of N-benzyl-N-β-phenylisopropyl-β-chlorethylamine HCl (drug 194) infused intravenously during 13 minutes. Bu-85-O: 1 mgm. per kgm. of Butanephrine HCl. The first number indicates minutes after the adrenolytic agent, drug 194; the second number indicates minutes after the tapenolytic agent, Butanephrine HCl.

A reversible tapenolysis appeared, shown by a pressor response to epinephrine (E-114-29) and the absence of a depressor response to Butanephrine (B-136-51) with a later reversion to depressor responses to both amines.

Experiments were performed to determine the tapenolytic effect of different amines. If epinephrine was employed as the test compound, the most effective tapenolytic drugs were the isopropyl homologue of epinephrine (Isuprel), ethylnorepinephrine (Butanephrine) and methylaminoacetocatechol (Kephrine). After apparently complete adrenolysis by drug 194, a large dose of any one of the three tapenolytic drugs reversed the response to epinephrine from depressor to pressor and the depressor effect of Isuprel or Butanephrine was abolished. Kephrine, at least after Isuprel, resembled epinephrine and became a pressor agent. A representative experiment in which Butanephrine produced tapenolysis is reproduced in figure 1. After the adrenolytic drug, 194, epinephrine produced only a depressor effect and the depressor response to Butanephrine appeared to be more pronounced. A very large dose of Butanephrine (1 mgm. per kgm.) was followed by the standard dose of epinephrine which then caused only a rise of blood pressure whereas Butanephrine was practically without effect. The progressive decline of tapenolysis during the last 99 minutes following the injection of the large dose of Butanephrine and the reappearance of depressor responses to the standard small doses of epinephrine and Butanephrine are shown in the remaining sections of figure 1.

Other experiments performed in cats are summarized in table 1. In most of these experiments 15–25 μM per kgm. of drug 194 was used as the adrenolytic agent. Dibenamine (68 μM per kgm.) and the methane sulfonates of dihydroergocristine or dihydroergocryptine (5 mgm. per kgm.) were similarly employed in several experiments. There are listed only those experiments in which "adrenolysis" could be demonstrated before the injection of the tapenolytic dose of amine. Isuprel, Butanephrine and Kephrine each could act as a tapenolytic agent: after the administration of a large dose, a subsequent small dose of epinephrine (or Kephrine) evoked a pressor response instead of the depressor action previously manifested. When tapenolysis had been produced by a large dose of epinephrine, small doses of epinephrine caused no change in blood pressure in contrast with the pressor response after tapenolytic doses of Isuprel, Butanephrine or Kephrine. Depressor amines like Isuprel or Butanephrine produced no response, provided that the corresponding drug was used as the tapenolytic agent. (Isuprel was also shown to produce tapenolysis of Butanephrine.) Norepinephrine, methylnorepinephrine (Cobefrine), desoxyephedrine or catechol in large doses did not lessen the depressor response to epinephrine after an adrenolytic drug. However, the pressor effect of small doses of norepinephrine was reduced after a large dose of the same drug had been administered.

Biörck (2) stated, without further comment, "In the experiments with low doses of dibenzyl-β-chloroethylamine there was some evidence, that large amounts of adrenaline were able to overcome and destroy the blocking effect, whereafter smaller doses could again manifest their normal excitatory effect." In our experience, "smaller" doses are then without any action, either pressor or depressor, and the large dose of epinephrine appears to have caused a temporary tapenolysis *and* adrenolysis. Peculiarly enough, epinephrine in small doses (e.g. 6 microgm. per kgm.) may provoke a temporary pressor response after doses

of 1–2 mgm. per kgm. each of epinephrine and desoxyephedrine (4 experiments) or of epinephrine and Vonedrine (1-methylamino-2-phenylpropane, 1 experiment).

In eight different experiments, not listed in the table, norepinephrine or Cobefrine (methylnorepinephrine) or both were injected after a tapenolytic dose of Isuprel, Butanephrine or epinephrine. In seven of these experiments, the test

TABLE 1

Tapenolytic effect of sympathomimetic amines after an adrenolytic drug

NO. OF EXPER.	TAPENOLYTIC AGENT		DRUG* TESTED FOR TAPENOLYSIS	RESULT
	Drug	Dose		
		mgm./kgm.		
9	Isuprel	1–2	Epinephrine	Tapenolysis with pressor response
2	Isuprel	1	Epinephrine	Incomplete tapenolysis only
2	Isuprel	2	Isuprel	Tapenolysis
1	Isuprel	1	Butanephrine	Tapenolysis
			Kephrine†	Tapenolysis with pressor response
2	Butanephrine	1	Epinephrine	Tapenolysis with pressor response
1	Butanephrine	1	Epinephrine	No tapenolysis
1	Butanephrine	1	Butanephrine‡	Tapenolysis
2	Kephrine	2	Epinephrine	Tapenolysis with pressor response
4	Epinephrine	1–2	Epinephrine	Tapenolysis only
2	Epinephrine	2	Isuprel	No tapenolysis
1	Norepinephrine	1	Epinephrine	No tapenolysis
			Isuprel	No tapenolysis
			Norepinephrine	Reduced pressor response
1	Cobefrine	1	Epinephrine	No tapenolysis
			Cobefrine§	Diphasic response persisted
2	Desoxyephedrine	2	Epinephrine	No tapenolysis
			Norepinephrine	Pressor response persisted
			Cobefrine	Pressor response persisted
1	Catechol	12	Epinephrine	No tapenolysis
			Norepinephrine	Pressor response persisted
			Cobefrine	Pressor response persisted

* All doses of 6 microgm. per kgm. unless noted otherwise.
† Dose of 14 microgm. per kgm.
‡ Dose of 4 microgm. per kgm.
§ Dose of 20 microgm. per kgm.

amines (norepinephrine and Cobefrine) still evoked a pressor response after the adrenolytic drug had caused epinephrine-reversal and this pressor response was not significantly modified by the large dose of tapenolytic drug. In the eighth experiment in which epinephrine was the tapenolytic agent and both test amines caused a rarely observed definite depressor effect before the large dose of epinephrine was given, tapenolysis without a pressor response occurred.

Discussion. The term tapenolysis is used to describe the blocking of a depressor response to a sympathomimetic amine. It is best revealed after an

adrenolytic drug has been administered when the customary response to epinephrine or Kephrine is depressor. If a large dose of Isuprel or Butanephrine is then injected, the depressor response to small doses of Isuprel or Butanephrine is abolished and a reversal from a depressor to a pressor response to epinephrine or Kephrine occurs. If epinephrine in a large dose is the tapenolytic drug, small doses of epinephrine do not affect the blood pressure until tapenolysis recedes when a depressor response reappears. The tapenolytic effect is reversible. No tapenolytic action toward depressor doses of epinephrine could be demonstrated after large doses of norepinephrine, Cobefrine, desoxyephedrine or catechol. Isuprel, Butanephrine and Kephrine have striking depressor effects in normal anesthetized cats and are the best tapenolytic agents. Epinephrine's depressor action is most easily demonstrated after an adrenolytic drug and it is probably a much less effective agent for blocking depressor responses than the other three.

The following hypothesis is suggested as an explanation of tapenolysis and the temporary reappearance of a pressor response to epinephrine after an adrenolytic drug and atropine sulfate. Vascular smooth muscle is furnished with separate and distinct sympathetic "reactive patches", one excitatory, the other inhibitory. Some drugs temporarily combine nearly exclusively with excitatory patches (e.g. norepinephrine), others with both types of patches although normal responses are usually excitatory (e.g. epinephrine), while the third type combines only with inhibitory patches (e.g. Isuprel). After a dose of adrenolytic drug which probably blocks most, but *not all*, excitatory patches and no inhibitory patches, the only effect of epinephrine which will ordinarily be recognized is inhibitory. A very large dose of a drug like Isuprel, combining with inhibitory vascular reactive patches remains loosely combined for many minutes during which Isuprel itself as well as epinephrine cannot gain access to such patches so that Isuprel is without action and epinephrine can cause a pressor response as it still has access to excitatory patches which have escaped the adrenolytic drug. Gradually the loose combination of Isuprel and inhibitory patches breaks down so that the latter are again free to react to small doses of Isuprel or epinephrine both of which cause the depressor responses present before the large tapenolytic dose of Isuprel had been given. If a large dose of epinephrine is given, not only is there tapenolysis but also an adrenolysis of excitatory patches which had escaped the blocking action of the first adrenolytic drug so that small doses of epinephrine temporarily are without any action and there later appears only the predominant depressor action previously present. (Contrary to our results, Biörck (2) reported a "normal excitatory effect" from small doses of epinephrine after the large dose.)

Undoubtedly such an hypothesis is only a simplified picture of events in particular experiments. Epinephrine in a large dose may have no effect on the pressor action of norepinephrine or Cobefrine. Also, although tapenolytic doses of epinephrine block inhibitory as well as excitatory responses of subsequent small doses of epinephrine, they appear not to block the inhibitory response to small doses of Isuprel; however, a still larger dose of epinephrine would be expected to block the Isuprel response. It is suggested that epinephrine, although

bound both to excitatory and to inhibitory reactive patches, may be so quickly displaced and replaced by amines such as norepinephrine or Isuprel that these substances can cause their characteristic responses. Thus after a very large dose of epinephrine, although small doses of epinephrine produce no response, doses of norepinephrine or Isuprel cause respectively a pressor or a depressor response.

SUMMARY

After an adrenolytic drug, the depressor response to epinephrine can be temporarily transformed into a pressor response if a large dose of a sympathetic depressor amine such as the isopropyl homologue of epinephrine (Isuprel) or ethylnorepinephrine (Butanephrine) first be given. Simultaneously, there is no vascular response to previously effective doses of the particular depressor amine used. This blocking of depressor responses is termed tapenolysis (sympathotapenolysis in a strict sense).

Experiments with other sympathomimetic amines are described. The best tapenolytic agents are Isuprel and Butanephrine. A large dose of epinephrine can be both tapenolytic and adrenolytic. An explanation of tapenolysis is offered.

Acknowledgements. We are greatly indebted to the following persons and their firms for drugs: Dibenamine (Dr. W. Gump and the Givaudan-Delawanna Corp.), Dihydro-ergot alkaloids (Dr. C. Henze and the Sandoz Co.), Drug 1974 and sympathomimetic amines (Mr. William Kirsch and the Smith, Kline and French Laboratories) and norepinephrine, Butanephrine, Kephrine and Cobefrine (Dr. M. L. Tainter and the Sterling-Winthrop Research Institute).

REFERENCES

1. Coret, I. A.: Proc. Soc. Exper. Biol. and Med., 68: 553, 1948.
2. Biörck, G.: Acta Physiol. Scand., 14: 174, 1947.

EFFECTS OF FEEDING URANIUM NITRATE HEXAHYDRATE IN THE DIETS OF BREEDING WHITE RATS[1]

ELLIOTT A. MAYNARD, CHALLISS RANDALL, HAROLD C. HODGE, AND JAMES K. SCOTT

Division of Pharmacology and Toxicology, Department of Radiation Biology, School of Medicine and Dentistry, University of Rochester, Rochester 7, New York

Received for publication December 1, 1948

MacNider, Helms, and Helms (1) observed the course of uranium nitrate poisoning in pregnant dogs. They found pathological changes in liver and kidney tissues and described alterations in certain constituents of blood and urine. Surviving dogs apparently experienced no serious interference with the normal completion of gestation. No other observations on the relationship between uranium poisoning and reproduction have come to our attention.

There is a double interest in the possible effects of uranium on the reproductive organs—first, because it is a highly toxic heavy metal; and second, because it is radioactive. The whole problem of interference with reproduction following exposures to uranium compounds seemed of sufficient importance to warrant at least some additional exploratory investigations. A study of the pattern of reproduction in rats fed uranium appeared to be a reasonable first step.

Experimental Methods. The general plan of this experiment was to observe the effects on reproduction when weanling rats (aged 28 days) were maintained in pairs on diets containing 2 per cent uranium nitrate hexahydrate. A similar group of litter-mated rats were paired in the same fashion to serve as a control group. The basic diet was purina fox chow meal with meat, a product of the Ralston-Purina Company, St. Louis, Missouri. Albino rats originally of the Wistar strain were used. Fifty males and 50 females were distributed in such a way that equal numbers of litter mates appeared in the control and in the experimental groups, but siblings were not mated. The females were separated from the males late in pregnancy. Litters were removed on the day of birth, and the females were returned to the males.

Effect on growth. From the body weight curves (figure 1), it is plain that the growth rate was noticeably depressed for both male and female rats fed 2 per cent uranium nitrate hexahydrate. At the end of about 7 months, the experimental male rats averaged about 65 grams less, the experimental female rats averaged about 60 grams less than their respective control groups. From food consumption measurements on other rats maintained on the same diets, it is known that the growth depression in the first 30 days is to be attributed mostly to a refusal of food (2); data on later periods are lacking.

Number and size of litters. Forty-four of the 45 control females had at least one litter; in sharp contrast, only 30 of 44 females in the experimental groups had litters (tables I and II). A careful inspection of table I will show that there was a constantly diminishing ratio of the number of litters in the experimental

[1] This paper is based on work performed under contract with the Manhattan District (United States Engineers' Office) at the University of Rochester, Rochester, New York.

group to those in the control group as time went on. The number of young differed in a regular and striking fashion between the control and experimental

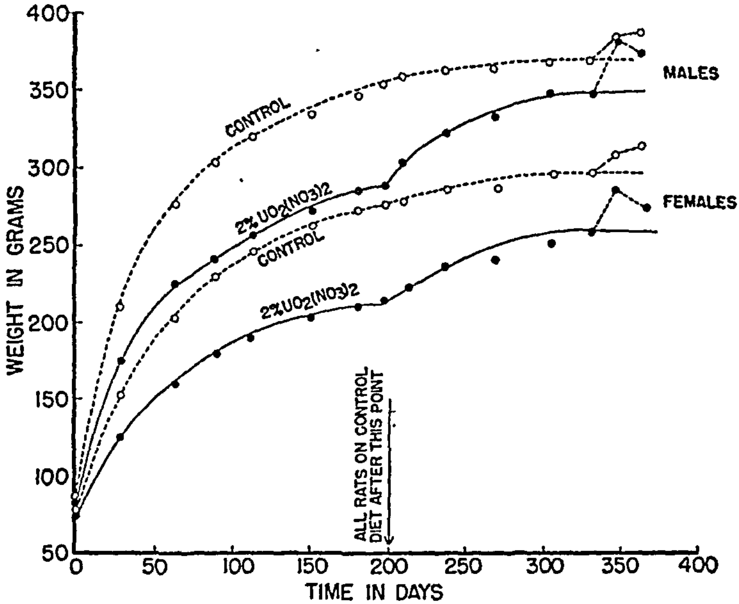


FIG. 1. GROWTH CURVES

For both male and female rats the growth curves of the experimental group maintained on a diet containing 2 per cent of uranium nitrate hexahydrate were depressed below the growth curves of the control groups, respectively.

TABLE I

Record of litters born in one-year experimental period

LITTER NUMBER	CONTROL RATS		EXPERIMENTAL RATS	
	No. having litters	Av. no. pups	No. having litters	Av. no. pups
1	44	7.9	30	7.4
2	43	9.9	28	7.9
3	41	9.7	26	7.7
4	39	9.7	25	8.6
5	36	9.2	24	8.3
6	35	8.2	23	8.7
7	33	8.5	18	7.3
8	30	7.9	15	8.4
9	25	7.5	13	6.8
10	19	5.7	9	6.2
11	14	5.6	2	7.0
12	5	6.0	—	—
13	1	11.0	—	—

groups (figure 2). After 216 days on the experimental regimen, a total of 2274 young had been born to control females, whereas 1049 young had been born to experimental females.

It is apparent that when rats are maintained on diets containing 2 per cent uranium nitrate hexahydrate, there is a marked decrease a) in the number of females having litters, b) in the number of litters, and, thus, c) in the number of young.

TABLE II

Litter record of rats

GROUP	NUMBER OF FEMALES	NUMBER OF LITTERS	AV. NO. LITTERS PER FEMALE	PUPS	AV. NO. PUPS PER FEMALE	AV. NO. PUPS PER LITTER
A. *During 216 days on diet containing 2 per cent uranium nitrate hexahydrate*						
Control...........	44	248	5.6	2274	51.7	9.2
Experimental.....	30	132	4.4	1049	33.0	7.9
B. *During 149-day period after removal of uranium nitrate hexahydrate from the diet*						
Control..........	34	116	3.4	823	24.2	7.1
Experimental....	23	82	3.6	638	27.7	7.8

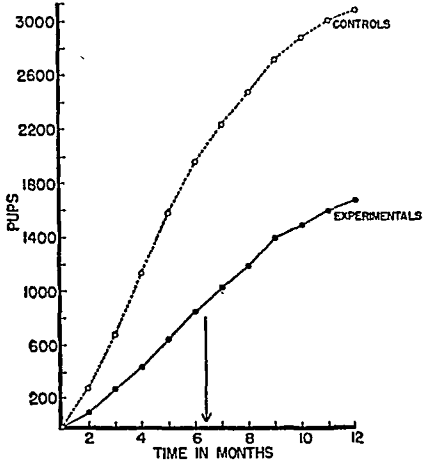


FIG. 2. OVERALL BIRTH RATE

T... ...e number born
to ... hexahydrate.
Wi...

Replacement on stock diet. During 195 days on the experimental diet, the patterns of growth reduction and of limitation in reproduction had become fixed. To determine whether these toxic effects were irreversible, the experimental animals were placed on the stock ration (i.e., no more uranium nitrate was fed),

and the experiment was continued for an additional 170 days. As may be seen from figure 1, there was an immediate resumption of growth both by the male and by the female experimental rats. The increased growth was insufficient to overcome entirely the previously-established weight depression; however, at the end of the experiment the control and experimental rats were fairly comparable in average body weight.

During this period of nearly 5 months, 34 of the control females and 23 of the experimental females had litters (table IIB). It is evident that the control animals continued their numerical advantage; however, on statistical grounds—that is, per female—the experimental animals were slightly more productive than the controls (figure 3). Specifically, in this period the experimental rats had about 5 per cent more litters per capita with about 15 per cent more young.

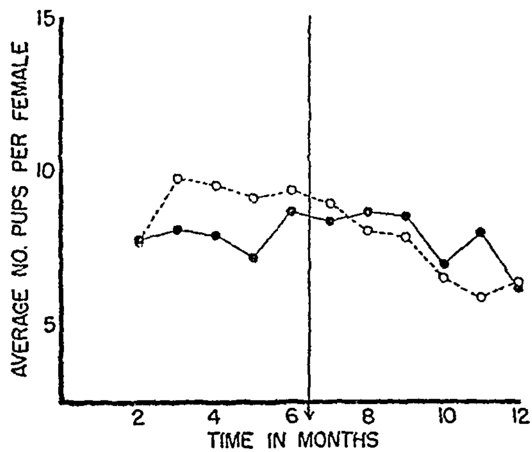


FIG. 3. PRODUCTIVITY OF PUPS PER FEMALE

During the period on the experimental diet, the average number of pups per female maintained on the experimental diet containing 2 per cent uranium nitrate hexahydrate (solid circles) was considerably below that of the control group females (open circles). When the experimental group was replaced on the stock ration (see arrow), the average number of pups per female thereafter tended to exceed the comparable production in the control group.

These figures represent a return to normal or above. It appears that the growth impetus and the health of those females which had litters were not irreparably depressed during the experimental period. It is noteworthy that the females which failed to have litters while ingesting uranium nitrate hexahydrate continued not to have litters during the 5 months on the stock diet.

Mortality. During the first 30 days on a diet containing 2 per cent of uranium nitrate hexahydrate, 4 females and 5 males died. Rats were removed from the control group to give a corresponding decrease in total numbers of animals under study.

During the remainder of the year, 3 females and 7 males in the control group died. In the same period 5 females and 3 males died in the experimental group. The overall mortality was therefore about 10 per cent for the control rats, a

figure which is close to the statistical mean (12 per cent) obtained at the end of a year on several thousand control animals from our colony. The slightly higher mortality (17 per cent) in the experimental group is in line with previous experience with rats on this diet.

Regularity of oestrus. When the loss of fertility shown in table II became apparent, the origin of the effect was sought. At the beginning of the 4th month of the experiment, daily microscopic studies were made of vaginal smears of each female rat using the technique of Long and Evans (3). From these observations it was shown that rats having regular oestrus cycles had regular matings (table III). Forty-one of the 45 control females had regular oestrus cycles accompanied by regular matings; in contrast, only 19 of 44 experimental females had regular matings and regular oestrus cycles.

Effects of reduced food consumption. Food consumption measurements on rats given a 2 per cent uranium nitrate hexahydrate diet at the same age have shown that a marked loss in appetite accompanied with a body-weight loss appeared in rats on the day following the first day of access to the uranium nitrate diet

TABLE III

Oestrus cycle regularity in relation to regularity of mating

	NUMBER OF FEMALES EXHIBITING			
	Regular oestrus cycles		Irregular oestrus cycles	
	Control	2% $UO_2(NO_3)_2$	Control	2% $UO_2(NO_3)_2$
Regular matings	41	19	0	0
Irregular matings	0	0	0	1
No matings	2	4	2	13
Totals	43	30	2	14

(2). The loss of appetite and loss of body weight continued in surviving rats for 5 to 12 days. When the rats began to eat again growth was promptly resumed; however, the body weight curves were permanently displaced below those of the control groups although the curves were practically parallel. Despite this fairly normal, although delayed, growth curve, the acute inanition in the first 2 weeks may have produced lasting injury to susceptible gonadal tissues (4).

Uranium content of young. On several occasions carcasses of newly-born rats were subjected to spectroscopic (5) or fluorophotometric (6) analysis for uranium. Despite the fact that the mother had been ingesting nearly 100 mgm. of uranium daily during the ingestion period[2], practically no uranium was found in the young. In some new-born, the content of uranium (fraction of a microgram) was such as to be just on the borderline of the ability of the analytical method to detect. Analyses of tissues taken from other adult female rats fed on the same 2 per

[2] The average rat in our colony eats 10 to 15 gm. of diet per day which would contain 200 to 300 mgm. of uranium nitrate hexahydrate (45 per cent U) or 90 to 135 mgm. of uranium per day.

cent uranium nitrate hexahydrate diet for comparable periods have shown consistent values of the order of 120 microgm. U/gm. bone, 16 microgm. U/gm. kidney and little in other organs or tissues (7). The percentage of the soluble uranium in the diet which is absorbed and enters the blood stream is minute indeed—a small fraction of one per cent (8). It seems a safe generalization that uranium (except in traces) does not cross the placental barrier. An additional factor which may account in part for the very low uranium content of the fetuses is the lack in them of sites for uranium deposition. In the adult, bone constitutes an important and permanent repository for uranium. In the fetus, the bone mineral is lacking and therefore no collection of uranium takes place.

Pathology. Although histological examinations were not made on the tissues of the rats in this experiment, such large numbers of both male and female rats have been studied with such care (9) that a general description of the pathological changes (renal and testicular) can be given for rats treated as these were.

In the kidneys, during the first week on a 2 per cent uranium nitrate hexahydrate diet, degenerative changes, necrosis and slough of the tubular cells occurred. At this time regeneration had begun. By the fourth to sixth weeks, both "typical" and "atypical" cellular regeneration were observed (10). During this period only a few necrotic or sloughed cells were found and the tubules were lined by regenerated epithelium. By the eighth to fourteenth weeks, in addition to the regenerating epithelium, some tubular atrophy and apparent increase in stroma were noted. These lesions persisted with little change during the remainder of the year's experiment. Rats that were fed a diet containing 2 per cent uranium nitrate hexahydrate for periods of 6 to 7 months and were then placed on a uranium-free stock diet for approximately the same length of time showed no appreciable renal abnormality.

Testicular lesions occurred following the feeding of uranium nitrate (2 per cent of diet) for one year. All the testes examined of rats on this diet showed degeneration and atrophic changes of variable degree. These consisted of a loss of spermatozoa in some tubules, absence of spermatids in others and loss of primary spermatocytes in others. In the last type of tubule only the Sertoli cells and a syncytium remained. In many tubules the lumens contained multinucleated cells. In some testes, variable numbers of tubules were lined by apparently normal germinal epithelium with adjacent tubules exhibiting degenerative changes; no normal tubules were observed in the sections of some testes. These changes were described by Dr. Karl E. Mason (11) as quite similar to those observed in general malnutrition or in vitamin E deficiency and different from the lesions produced by ionizing radiation (4). In the sections Dr. Mason examined, he found one of two characteristic pictures: a) complete degeneration of all tubules indistinguishable from that of advanced vitamin E deficiency or b) variable numbers of partially or completely degenerated tubules interspersed among essentially normal tubules, similar to the appearance when a mild deficiency of vitamin E had existed at the time of sexual maturity but had been corrected before all tubules were affected. There seemed to be sufficient normal sperm in the ducts to consider these males normal in reproductive capacity.

Discussion. When breeding rats are maintained on a diet containing 2 per cent of uranium nitrate hexahydrate, at least 3 effects are noted: a) there is a reduction in the number of females having litters, b) the oestrus cycles are mostly irregular, and c) the number of young per litter is smaller. The reduction in the number of fertile pairs may be in part ascribed to the injuries formed in the testes; what failure in the female, if any, has also occurred is not known. The irregularities in oestrus cycle cannot be a result of inanition in the ordinary sense; food consumption records show that such rats, after an initial period of reduced food intake, recover their appetites and consume practically normal amounts of the diets. Peculiarly, the falling off in number of pups per litter that is a usual occurrence in our colony when the breeding females reach ages beyond 8 months was not seen in the experimental rats given the stock ration at about that age. Females in the experimental group, which had failed to have litters during the period on the uranium nitrate diet did not develop the ability to bear litters when transferred to the stock diet. Females in this group had failed to exhibit the normal increase in number of pups per litter early in the study: control group—1st litter, 7.9, 2nd through 5th litters, 9.9 to 9.2; experimental group—1st litter, 7.4, 2nd through 5th litters, 7.9 to 8.3 pups per litter. That no serious injury had been suffered by the reproductive systems that functioned is shown by the number of pups per litter in the last phase of the experiment; the 9th, 10th and 11th litters averaged 7.5, 5.7 and 5.6 for the control group against 6.8, 6.2 and 7.0 for the experimental group.

The question of radiation effects from possible uranium exposures in this test needs only small comment. From analyses of tissues and from distribution studies, it has been found that only minute traces of uranium reach the reproductive organs. It is improbable that the uranium unabsorbed from the diet and passing through the gastrointestinal canal could irradiate the vital gonadal tissues in a detectable fashion. The histological changes seen in the testes confirmed these assumptions since they were not the lesions known to follow irradiation.

Acknowledgment. The authors gratefully acknowledge the assistance of Dr. Karl E. Mason.

SUMMARY

1) Two groups of 50 pairs of rats each were maintained for 7 months on a control diet and on a diet containing 2 per cent of uranium nitrate hexahydrate, respectively.

2) The growth rate of both male and female rats receiving the 2 per cent uranium nitrate hexahydrate diet was notably depressed. An increased growth occurred, however, when the experimental rats were placed on the control diet 7 months after the start of the experiment.

3) Uranium feeding sharply reduced the number of female rats having litters. In addition, the experimental rats had fewer young per litter. When the experimental rats were replaced on the stock diet the females having litters were

more productive than the control rats. Females which failed to have litters on the experimental diet did not have litters later on the stock diet.

4) Rats fed 2 per cent uranium nitrate hexahydrate exhibited a slightly greater mortality during the 12-month period.

5) Uranium nitrate hexahydrate feeding produces an irregularity of oestrus cycle. Only 19 of 44 experimental female rats had regular oestrus cycles and regular matings.

6) Uranium, except in traces, does not cross the placental barrier.

REFERENCES

1. MacNider, W. deB., Helms, S. T. and Helms, S. C.: Bull. J. Hopkins Hospital, 40: 145, 1927.
2. Maynard, E. A., Hodge, H. C., Downs, W. L., Kesel, R., and Randall, C.: N.N.E.S., Div. VI, Book I—Pharmacology, Volume I, Chapter VII, Section 3 (In press).
3. Long, J. A., and Evans, H. M.: Mem. U. Calif., 6: 19, 1922.
4. Mason, K. E.: Am. J. Anat., 52: 133, 1933.
5. Steadman, L. T.: N.N.E.S., Div. VI, Book I—Pharmacology, Volume II, Chapter II, Section 4 (In preparation).
6. Neuman, W. F., Fleming, R. W., Carlson, A. B., and Glover, N.: J. Biol. Chem., 173: 41, 1948.
7. Neuman, W. F.: N.N.E.S., Div. VI, Book I—Pharmacology, Volume II, Chapter III (In preparation).
8. Goodenough, A., Barnett, T. B., and Hodge, H. C.: N.N.E.S., Div. VI, Book I—Pharmacology, Volume II, Chapter III, Section 1 (In preparation).
9. Barnett, T. B., and Metcalf, R. G.: N.N.E.S., Div. VI, Book I—Pharmacology, Volume I, Chapter IV (In press).
10. MacNider, W. deB.: J. Exper. Med., 49: 387, 1929.
11. Mason, K. E.: personal communication.

PHARMACOLOGICAL STUDIES ON THE CAUSATIVE AGENT OF CANINE HYSTERIA

JACK L. RADOMSKI[1] AND GEOFFREY WOODARD

Division of Pharmacology, Food and Drug Administration, Federal Security Agency, Washington, D. C.

Received for publication December 1, 1948

Recent work in this and other laboratories has established that a reaction product of nitrogen trichloride, the active constituent of "agene", and the protein of wheat flour is the etiological agent of canine hysteria (1–5). The toxicity of this agent to other species besides the dog has been dealt with in previous papers (1, 3, 6). The failure of all investigators to obtain reproducible positive results with agene-treated amino acids and other constituents of flour (7) has led to the supposition that the toxic principle is an altered peptide. Attempts in this laboratory to isolate the toxic principle from enzymatic hydrolysates of agene-treated gluten have been so far unsuccessful. The apparent magnitude of the task of isolating one peptide from an enzymatic hydrolysate leaves considerable doubt as to how long it will be before the purified substance is available for pharmacological study. The techniques and experiments to be described represent preliminary attempts to learn something of the pharmacology of the toxic principle of this agene-treated protein using the most toxic material presently available. More specifically, the accumulation characteristics of the substance, its tendency to produce increased sensitivity on repeated administrations, possible synergists, and possible antagonists were investigated.

MATERIAL. The material used in all these experiments was commercial gum gluten (80 per cent protein)[2] which had been treated with 6 gm./kgm. of NCl_3. This level of treatment has been shown to yield a maximally toxic product (1).

RESULTS. *Accumulation characteristics.* A curve expressing the accumulation characteristics of this toxic agent has been determined. Six groups of 4 purebred Dalmatians each were administered in capsules agene-treated gluten in daily doses ranging from 0.05 to 2.0 gm./kgm./day. The average number of days required for the animals to develop running fits was plotted against the daily dosage level. The curve obtained, shown in figure 1, closely resembles a hyperbola (represented by the equation $(x - 1.5)(y - 0.07) = 1.24$) which is asymptotic to the two dotted lines, $y = 0.07$ and $x = 1.5$. The asymptote (dotted line) parallel to the x-axis represents the largest dose of agene-treated gluten which can be given for an indefinite period without producing fits. The value of this constant is 0.07 gm./kgm./day, the threshold daily dose above which accumulation will occur. The experiment was terminated after 146 days.

[1] Presented before the 38th meeting of the American Society for Pharmacology and Experimental Therapeutics, Inc., in Atlantic City, March 15–19, 1948, and revised to include further data.

[2] Keever Starch Company, Columbus, Ohio.

A similar dose-time curve to the one just described for dogs was likewise determined for rabbits. Groups of four rabbits were administered agene-treated gluten by stomach tube in daily doses of from 1 to 15 gm./kgm./day. Unfortunately, it was not possible to determine the threshold level for accumulation for rabbits as accurately as for the dogs because of the unfavorable effect of daily stomach tubings. At the 1 gm./kgm./day level, three animals survived for twenty days, none of which showed symptoms of intoxication. It can be estimated from figure 2 that the threshold level for accumulation is 0.5 to 1 gm./kgm./day. This is seven to fourteen times higher than the value for the dog, although the single effective doses are 10 gm./kgm. for rabbits and 3.5 gm./kgm. for dogs (1). Thus the ratio of acute toxicity to threshold of accumulation is much lower for the rabbit than for the dog.

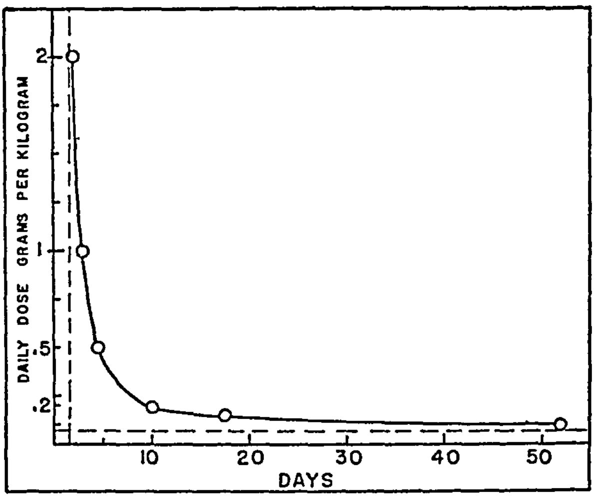


FIG. 1. DOSE-TIME CURVE FOR DOGS

The average time of appearance of fright fits in groups of 4 dogs vs. dosage of agene-treated gluten.

To elucidate further the accumulative characteristics of the toxic principle an additional experiment, the results of which are shown in figure 3, was carried out. Four Dalmatians were administered daily doses of 0.5 gm./kgm. of maximally toxic gluten until they developed running fits. The average number of daily doses for the production of fits is represented by the height of the first bar on the graph (5 days). Treatment was suspended for ten days after which time the same four dogs were again given the same daily dose until they came down with fits. The time required to produce fits on the second trial is the second bar on the graph (5 days). However, the third time this procedure was repeated following a 10-day suspension of treatment, the dogs came down with fits in only two or three days and the fourth time in two days. At this point treatment was discontinued for forty days after which toxic gluten was again administered as before. The length of time required to produce fits had re-

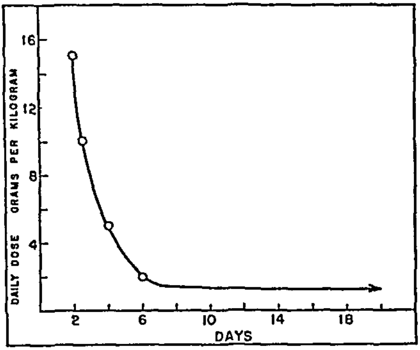


FIG. 2. DOSE-TIME CURVE FOR RABBITS
The average time of the appearance of fright fits in groups of 4 rabbits vs. dosage of agene-treated gluten.

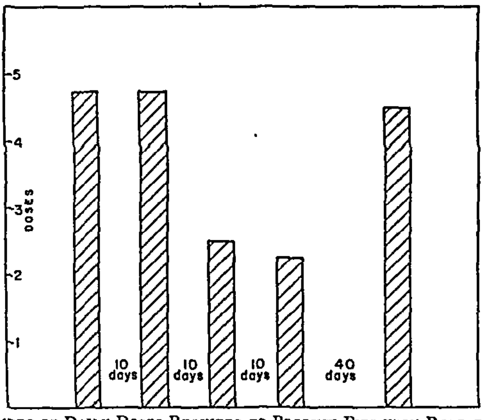


FIG. 3. NUMBER OF DAILY DOSES REQUIRED TO PRODUCE FITS UPON REPEATED PERIODS OF ADMINISTRATION OF AGENE-TREATED GLUTEN TO DOGS
The level of agene-treated gluten administered was 0.5 gm./kgm./day.

turned to normal as shown in the last bar on the graph. These results show that after ten days without receiving further amounts of treated gluten, a residue tending to produce increased sensitivity remained in these animals.

Synergists and antagonists. In order to provide a firmer basis for the comparison of canine hysteria with human idiopathic convulsions, as well as to

provide a clue to the site and mechanism of action of the etiological agent, the effects of certain pharmacological agents on this disorder were investigated. These agents included metrazol, ammonium chloride, physostigmine, parathion, glutamic acid, and the anti-convulsants, diphenylhydantoin sodium, trimethadione, and phenobarbital. The effect of extreme hydration was also investigated.

The use of metrazol in subconvulsive doses has been often recommended as a test for the diagnosis of epilepsy. Romano has reported (7) that the threshold for metrazol convulsions was consistently lower in epileptics than in normal individuals. In order to apply this test to canine hysteria, it was first necessary to determine an intravenous dose of metrazol that was just subconvulsive to dogs. This dose was found to be 10 mgm./kgm. of metrazol in 10 per cent solution injected intravenously as rapidly as is practical. This dose did not produce a convulsive reaction in ten normal dogs; a dose of 12.6 mgm./kgm.

TABLE 1

Metrazol synergism

GLUTEN ADMINISTRATION		CONDITION OF DOGS	DOSE OF METRAZOL	POSITIVE REACTION
Dose	No. of daily doses			
gm./kgm.			*mgm./kgm.*	
—	—	Normal	10	0/10
—	—	Normal	12.6	1/10
1*	12	Ataxic	10	8/ 8
1*	20	Very ataxic	6	2/ 3
2	1	Not affected	10	0/ 3
3	1	Ataxic	10	3/ 3
5	1	Recovered†	10	1/ 2

* The gluten used in these cases was considerably less potent than the standard 6 gm./kgm. agene-treated gluten.

† Tested 8 days after gluten administration was discontinued.

produced only one reaction in ten dogs. However, when injected into eight dogs which had received 12 daily doses of agene-treated gluten (sufficient to produce slight to moderate ataxia) a 10 mgm./kgm. dose of metrazol produced a distinct positive reaction in each case (see table 1). Furthermore, two of three dogs receiving the same dose of gluten for 20 days reacted to a lower dose of metrazol, 6 mgm./kgm. The reactions varied from typical metrazol convulsions to typical running fits. The usual reaction appeared to be a mixture of metrazol symptoms and the fright fit syndrome. After a latency of 15 seconds, the animal usually was seized with a metrazol-ty[illegible]nvulsion of brief duration which was normally succeeded by a typic[illegible] fit and/or a [illegible]convulsion. The recurrence at irregular interval[illegible] of these epis[illegible] frequent up to ½ hour after the injection. [illegible]iod, [illegible] in [illegible] usually be precipitated [illegible] sound [illegible] reacti[illegible]ct contrast to a simpl[illegible] convulsi[illegible]lorly t[illegible]ic and with low doses [illegible]

The above described metrazol test was also administered to dogs receiving single doses of agene-treated gluten. At 3 gm./kgm. which produced distinct ataxia but no fits, all three dogs reacted positively while at 2 gm./kgm., none of the three dogs tested reacted. One of two dogs which had apparently recovered from a 5 gm./kgm. dose of gluten administered eight days previously also reacted positively to the test.

Another clinical test for latent epilepsy is the hydration test (8, 9). This test was developed on the basis of experimental work demonstrating that excessive tissue hydration lowers the convulsive threshold of animals (10). Using the procedure recommended for dogs by Allen (11) involving the administration of five doses at $\frac{1}{2}$-hour intervals of 300–500 cc. of water, eight animals receiving sub-effective daily doses of gluten were tested. Of these, only one animal developed a fit or convulsion.

It is well known that systemic acidosis tends to prevent petit mal attacks while alkalosis tends to precipitate them. An experiment intending to establish whether canine hysteria is similarly affected was done. Three groups of four pure-bred Dalmatians each were given a daily dose of 1 gm./kgm. of agene-treated gluten. Simultaneously one of the groups was administered 5 gm. of ammonium chloride three times a day in enteric-coated capsules to produce acidosis and the second group was administered an identical dose of sodium citrate to produce alkalosis, the third group serving as controls. Intermittent determinations of urine pH showed that the desired shifts of the acid-base balance had been achieved. No significant difference as to the appearance of the first fit or frequency of fits among the three groups was observed.

One of the prevalent theories of the etiology of some human convulsive disorders involves the production of an excessive concentration of acetylcholine in the brain (12). However, when subconvulsive doses of physostigmine were administered to four dogs which had been pre-treated with agene-treated gluten, no synergism was observed. Nor was an effect obtained with subconvulsive oral doses of gluten and parathion administered simultaneously to another group of four dogs. Both physostigmine and parathion are known to inhibit brain cholinesterase.

Antagonists. In a search for pharmacological antagonists of canine hysteria, the effects of the common anti-convulsant drugs, diphenylhydantoin sodium, trimethadione and phenobarbital were investigated. These drugs were administered in divided doses every day for a week, the total daily dose appearing in column one in table 2. Groups of four pure-bred Dalmatians each with litter mates randomized across the groups were used. On the second day of the experiment single doses of 5 gm./kgm. agene-treated gluten were given by capsule to all dogs. The results obtained are shown in table 2. Surprisingly, diphenylhydantoin and to a lesser extent trimethadione aggravated the effect of agene-treated gluten instead of antagonizing it, for instead of mild, running fits the dogs receiving these drugs in several cases manifested convulsions severe enough to produce death. While the dose of phenobarbital administered produced a considerable degree of depression, it did suppress the convulsions.

The unexpectedness of the diphenylhydantoin result in particular stimulated further experimentation with this substance. An experiment similar to the ones described above showed that the potentiation effect did not persist to a lower 2 gm./kgm. dose of gluten. A further experiment involving six dogs in which a daily dose of 0.2 gm. was given for three weeks along with 20 mgm./kgm./day of diphenylhydantoin showed no effect of the drug, either antagonistic or synergistic.

TABLE 2

Effects of some anti-convulsants on canine hysteria

	DAILY DOSE	RESULTS		
		Fits	Convulsions	Deaths
	mgm./kgm.			
Control	—	4/4	0/4	0/4
Diphenylhydantoin	60	4/4	4/4	3/4
	10	4/4	2/4	2/4
Trimethadione	150	4/4	3/4	2/4
Phenobarbital	60	0/4	0/4	0/4

TABLE 3

Effect of phenobarbital on canine hysteria

	DOG NO.	SEX	NUMBER OF DAYS‡						
			0–9	10	12	14	16	18	20
Agene-treated gluten, 0.5 gm./kgm./day	1	M	—*	Fits	Fits	Conv. dead			
	2	M	—	Conv.	Conv.	Fits	Fits	—	—
	3	F	—	Conv.	Dead				
	4	F	—	—	Fits dead				
Agene-treated gluten, 0.5 gm./kgm./day and phenobarbital§, 20 mgm./kgm./day	1	M	—	—	—	—	—	—	Conv. dead
	2	M	—	—	—	—	—	—	Conv. dead
	3	F	—	—	—	—	—	—	Fits
	4	F	—	Fit†					

* A dash (—) indicates no effects observed.

† Mild fit after which dog was removed from experiment.

‡ Agene-treated gluten administration discontinued on thirteenth day.

§ Phenobarbital administration discontinued on seventeenth day.

It was thought that an experiment in which agene-treated gluten would be administered in daily subacute doses and phenobarbital in sub-depressant daily doses would more clearly reveal the action of this anti-convulsant on canine hysteria. Accordingly, a litter of eight dogs was divided into two groups. Both groups of dogs were administered 0.5 gm./kgm./day of agene-treated gluten, and in addition one group was simultaneously administered phenobarbital at a level of 20 mgm./kgm./day in two daily doses. The results obtained are shown in table 3. By the thirteenth day all of the controls had shown fits and convulsions,

leading in three cases to deaths due to the administration of agene-treated gluten while only one of the experimental dogs showed any effect. This one dog showed a mild fit on the tenth day and the administration of both the gluten and the phenobarbital was stopped. No gluten was given past the thirteenth day to the remaining seven dogs and the phenobarbital was discontinued in the three experimental animals on the sixteenth day. Four days later these three dogs developed fits and convulsions leading to deaths in two cases. It should be emphasized that the fits and convulsions in the three experimental animals occurred only after the effects of the phenobarbital had worn off but while the animals still retained residual effects from the earlier discontinued gluten administration. Thus the antagonism of phenobarbital against the ability of agene-treated gluten to produce fits, convulsions, and deaths in dogs has been clearly demonstrated.

Clinical results showing that glutamic acid suppresses petit mal (13) led to a preliminary experiment on 2 dogs with simultaneous oral administration of this substance and agene-treated gluten. No suppressive effect of the glutamic acid was observed.

DISCUSSION. Several of the properties of the dose-time curves presented are worthy of note. The fact that the curve becomes asymptotic to the line $y = 0.07$ shows that the dog has a limited maximum capacity for handling the toxic principle, for within the range of doses employed the amount destroyed and/or excreted was not proportional to dose but instead a constant independent of dose. However, this threshold for the rabbit is apparently even higher than its greater resistance to an acute dose would lead one to expect, which indicates that a relatively high level of detoxification is taking place. The asymptote parallel to the y-axis of the curve for the dog is the minimum time of onset of this disorder, i.e., fright fits. That this is as long as 1.5 days is an argument in favor of the thesis that it is a tissue alteration that is cumulative rather than the substance itself. This thesis is also supported by the observation that a period longer than ten days is necessary for complete recovery of the animal, as evidenced by a return to his original sensitivity. If the toxic principle is in fact a peptide, it seems reasonable that it would be built into a proteinatious constituent of the body, for example, the tissue protoplasm at the sight of action thereby forming a functional lesion in, presumably, the brain.

The results obtained with metrazol demonstrate that the convulsive threshold to metrazol was lowered by the previous administration of agene-treated gluten. Further, the response obtained with a combination of these two substances was a combination of the two syndromes. Taken together, these results strongly suggest that these two central nervous system stimulants have a site or a mechanism of action in common.

The results obtained with the anti-convulsants tested clearly demonstrate that diphenylhydantoin and trimethadione do not antagonize the syndrome produced by agene-treated gluten. The aggravating effect of diphenylhydantoin in large doses is interesting, though at present inexplicable. On the other hand, phenobarbital clearly antagonizes canine hysteria.

Sufficient information has been gathered about the properties of canine hysteria to compare this pathological entity with human epilepsy; table 4 contains such a comparison. No attempt is made to differentiate between the various types of epilepsy, or to draw a conclusion from the comparison.

TABLE 4

A comparison of the properties of canine hysteria and human epilepsy

EPILEPSY	CANINE HYSTERIA
1. Spontaneous seizures at intervals.	1. Spontaneous seizures at intervals.
2. Periods of normality between fits.	2. Ataxia always evident between fits.
3. Abnormal electroencephalograph.	3. Abnormal electroencephalograph.
4. No hysteria.	4. Hysteria.
5. Precipitated by hyperventilation, alkalosis, and hydration and suppressed by the opposite.	5. Not affected by any of these conditions.
6. Subnormal threshold to metrazol.	6. Subnormal threshold to metrazol.
7. Suppressed by diphenylhydantoin or trimethadione.	7. Not suppressed by diphenylhydantoin or trimethadione.
8. Suppressed by phenobarbital.	8. Suppressed by phenobarbital.

SUMMARY

1. The dose-time curve of agene-treated gluten was found to resemble a hyperbola. The value for the threshold daily dose for accumulation to occur was found to be 70 mgm./kgm. for the dog and approximately 0.5 to 1.0 gm./kgm. or seven to fourteen times as great for the rabbit. These may be compared to acute toxicity values of 3.5 gm./kgm. for the dog and 10 gm./kgm. for the rabbit.

2. A considerable residue of the toxic effect tending to produce increased sensitivity on repeated administration remained after an interval of ten days.

3. The previous administration of agene-treated gluten in doses sufficient to produce ataxia lowers the threshold of reaction to metrazol. This reaction has properties of both canine hysteria and metrazol convulsion.

4. Hydration produced by the administration of water does not appear to precipitate fits in dogs administered sub-effective doses of agene-treated gluten.

5. Neither the administration of ammonium chloride nor sodium citrate affects the production of canine hysteria by agene-treated gluten.

6. Neither diphenylhydantoin nor trimethadione suppresses canine hysteria. Diphenylhydantoin and possibly also trimethadione potentiate the production of convulsions and death by agene-treated gluten.

7. Phenobarbital antagonizes the production of canine hysteria.

REFERENCES

1. Radomski, J. L., Woodard, G., and Lehman, A. J.: J. Nutrition, 36: 15, 1948.
2. Moran, T.: Lancet, Aug. 23, 1947, p. 289.
3. Newell, G. W., Erickson, T. C., Gilson, W. E., Gershoff, S. N., and Elvehjem, C. A.: J. A. M. A., 135: 760, 1947.

4. Silver, M. L., Johnson, R. E., Kark, R. M., Klein, J. R., Monahan, E. P., and Zevin, S. S.: J. A. M. A., 135: 757, 1947.
5. Mellanby, E.: Brit. Med. J., II: 885, Dec. 14, 1946.
6. Mellanby, E.: Brit. Med. J., II: 288, Aug. 23, 1947.
7. Newell, G. W., Gershoff, S. N., Fung, F. H., and Elvehjem, C. A.: Am. J. Physiol., 152: 637, 1948.
8. Romano, J.: La Semana Medica, 50: 310, 1943.
9. Blyth, T.: Brit. Med. J., I: 100, 1943.
10. Swinyard, E. A., Toman, J. E. P., and Goodman, L. S.: J. Neurophysiol., 9: 47, 1946.
11. Allen, F. M.: Am. J. Psychiatry, 102: 67, 1945.
12. Forster, F. M.: Arch. Neurol. & Psychiatry, 51: 393, 1944.
13. Waelsch, H., and Price, J. C.: Arch. Neurol. & Psychiatry, 51: 393, 1944.

THE EFFECT OF HYPOXIA ON THE IN VIVO FORMATION OF METHEMOGLOBIN BY ANILINE AND NITRITE

S. S. SPICER AND P. A. NEAL

Laboratory of Physical Biology, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda 14, Maryland

Received for publication December 3, 1948

A problem suggested by the anticipated use of aromatic amines as adjuvant in aviation gasoline has led to the incidental observation that under conditions of hypoxia the methemoglobinemic activity of certain of these compounds is attenuated. Xylidine, the compound regarded as a potential hazard to aviation personnel, was tested in pilot experiments for its methemoglobinogenic effect in dogs by skin application at simulated altitude and at ground level, but was found to produce little or no methemoglobin (MHb) under either condition. Because of the known ability of cats to react with methemoglobinemia to percutaneous xylidine (1), this species was then used with the result that less MHb was found at altitude than at ground level. In order to explore further the effect of hypoxia on MHb formation in dogs, administration of aniline by skin application and intravenous injection, and injection of aniline metabolic intermediates and sodium nitrite, was undertaken at normal and reduced pressures.

PROCEDURE. Before, and at appropriate intervals after administration of a test substance, blood samples obtained by venipuncture were assayed for MHb and total hemoglobin (Hb) content according to the method of Horecker and Brackett (2), using their apparatus or the simplified photometer of Andrews and Horecker (3). The 1:10 dilution of blood in distilled water or borate buffer was made as soon as the blood samples were drawn, thereby stabilizing the MHb concentration. A large decompression chamber having a ventilation rate of 25 cubic feet per minute was used to induce hypoxemia. Exposures to reduced pressure were carried out at 27,000 to 28,000 feet simulated altitude and lasted 4 to 6 hours; ascent to altitude took place at a rate of 6,000 feet per minute after intravenous (i.v.) injection. Exposure to lowered atmospheric pressure was accomplished as soon as possible after administration of the test substance except on one nitrite run and in tests with the "active intermediates" when injection was done and blood samples were drawn at simulated altitude. Samples were taken ordinarily in anoxia experiments by bringing the animals down abruptly and returning them to altitude as quickly as possible after venipuncture.

Except in the case of nitrosobenzene which was prepared in 50 per cent propylene glycol in water, all materials for injection were made up in water solution. Solutions to be injected were prepared fresh before each test and in an identical manner for ground and altitude runs using the same glassware and syringes for each. The animals were injected in the jugular vein, and whenever it appeared the solution was not injected entirely within the lumen of the vein, the test was discontinued.

RESULTS. I. *Intravenous aniline.* In preliminary experiments a marked difference was noted between normal and hypoxemic cats in the level of MHb attained after cutaneous application of xylidine. When administered in repeated small doses for several days, xylidine caused much less MHb in animals exposed simultaneously to reduced pressure than in controls at ambient pressure. When

xylidine was given to cats, or aniline administered to dogs in a single large cutaneous dose, the level of MHb attained was less in hypoxemic animals during but rose to concentrations attained in the controls following exposure to simulated altitude. The relative anoxemia appeared to have an inhibitory influence on the development of Heinz bodies (measured by the method of Horecker (4)) in cats given xylidine cutaneously, but not enough data was available to be certain of this point.

In order to determine the effect of anoxia on MHb formation independent of its influence on skin absorption, intravenous administration was employed. Fifteen mgm./kgm. of aniline[1] were given intravenously to three dogs at ground level on a number of occasions, and the MHb concentration was followed in blood samples

TABLE 1

Maximal per cent MHb following i.v. aniline 15 mgm./kgm.

Ground level

DOG	DATE													DOG	DATE			
	6/20	9/4	9/6	9/7	9/11	9/14	9/17	10/16	11/23	11/26	12/10	12/17	12/21		1/23	1/30	2/8	3/29
1....	20.2	25.8	29.6	25.9	28.3	22.1	26.7	21.3	34.6	27.3	25.7	30.4	24.6	4....	32.4	32.0	24.0	28.3
2....	19.7	27.0	25.0	27.3	27.0	21.2	26.1	28.8	28.6	27.9	26.4	25.8	27.0	5....	27.0		23.2	19.1
3....	36.0	33.7	38.6	37.9	37.3	30.5	39.6	41.5	39.7	37.4	30.1	38.1	32.9	6....	32.1	31.2	28.1	30.2

Altitude

DOG	DATE										DOG	DATE		
	6/15	6/23	9/20	10/15	10/18	10/19	10/22	10/31	11/19	11/28		1/2	2/4	2/7
1......	10.8	9.8	16.2	7.6	11.0	7.7		14.5		20.1	4.......	21.0	15.5	
2......	12.8	11.0		16.2	16.8	11.6	12.5		17.6		5.......	16.2		10.1
3......	22.6	14.7	32.0				15.7	19.6	26.0	17.7	6.......			8.3

taken at 30 to 45 minute intervals between 2 and 5 hours after injection. This process was repeated several times in the same animals with the difference that they were exposed 4 to 5 hours to 28,000 feet simulated altitude immediately following injection. The results of these tests and of a series of identical runs made on another group of 3 dogs are shown in table 1. Again less methemoglobin was found in anoxic animals than in ground controls. In table 2, it is seen that this applies, also, though to a lesser extent, to a dose of 30 mgm./kgm. An analysis of variance of the maximum per cent MHb in 13 ground runs on three dogs showed significant differences between days and highly significant differences between the three dogs. The mean of the maximal MHb percentages found after 15 mgm./kgm. for dogs 1, 2, and 3 was 26.3, 26.7, and 36.3, the least significant mean difference being 2.1 per cent. It was also evident that not only the MHb peak but also the time required for the MHb concentration to return to normal was greater for dog 3.

II. *Intravenous nitrite and aniline derivatives.* It seemed not unlikely that the

[1] Purified by distillation at 184° shortly before use.

impairment in aniline action at reduced pressure might be due to retardation of one or more of the oxidative steps by which aniline is converted to the active MHb-forming intermediate. In this case the methemoglobinemia from a compound such as $NaNO_2$ which acts stoichiometrically without metabolic alteration should not be inhibited by anoxia. In fact, it ought to be possible after determining the effect of anoxia on MHb production by the several compounds representing stages in aniline oxidation to demonstrate at what point hypoxia interferes in the ability of aniline to form MHb.

The procedure followed for the intravenous aniline experiments was carried out in the same manner in a series of runs giving sodium nitrite to three of the dogs. In most of the anoxia tests nitrite was injected at ground level and the

TABLE 2

Maximal per cent methemoglobin after i.v. aniline 30 mgm./kgm.

Ground Level

DATE			
2/9		4/10 and 4/12	
Dog 1	52.1	Dog 4	52.2
Dog 2	47.0	Dog 5	35.6
Dog 3	61.8	Dog 6	52.7

Altitude

DATE					
4/11		4/12		4/15	
Dog 1	40.6	Dog 3	54.3	Dog 3	52.7
Dog 2	36.8	Dog 4	44.4	Dog 5	25.5
				Dog 6	24.8

animals were taken to 28,000 feet within six minutes of the time of injection. The process of bringing them down for samples and returning to altitude usually required 7 minutes. Samples were taken at 20 to 40-minute intervals over the period $\frac{3}{4}$ to 2 hours after injection. A ground level and an altitude run were made administering nitrite to another group of three dogs, and in the latter the nitrite was injected and samples were taken at altitude. The data with nitrite which are summarized in table 3 show that at least as high a level of MHb, if not a slightly higher one, occurs at reduced pressure. The individual variation in maximal methemoglobinemia after aniline in which the mean for dog 3 was 37 per cent greater than that for dogs 2 and 3 was less obvious after nitrite when the mean for dog 3 was 9 and 2 per cent greater than that for dogs 1 and 2, respectively.

Several similar experiments were attempted administering the oxidation products of aniline: nitrosobenzene[2] and p-aminophenol. Here the injection

[2] Preparation of Edcan Laboratories, South Norwalk, Conn. (Received shortly before use.)

was made and blood samples were taken and diluted at reduced pressure. Nitrosobenzene was given at a dose of 2.5 mgm./kgm. or a total dose of 11.0 mgm. (dogs 1–6) at ground level and altitude. The dose of p-aminophenol was 10

TABLE 3

Maximal per cent MHb following i.v. sodium nitrite 17 mgm./kgm.

Ground Level

DOG	DATE								DOG*	DATE
	7/20	7/24	7/26 & 8/9	8/10	8/13	8/31	9/5 & 9/18	10/9		4/9
1.......	27.0	27.0	25.5	28.5	26.8	27.0	31.9	26.3	5A.......	25.9
2.......	26.9	26.3	29.6	29.3	33.8	30.0	30.9	27.8	6A.......	25.0
3.......	29.6	29.1	30.4	29.6	31.4	30.4		29.9	8A.......	39.1

Altitude

DOG	DATE				DOG*	DATE
	9/5	9/10	9/12	9/18		4/15
1..............	30.4				5A.............	24.6
2..............		31.0		34.5	6A.............	34.8
3..............			33.5	37.2	8A.............	39.5

* Dogs 5A, 6A, and 8A received 20 mgm./kgm. on both runs.

TABLE 4

Effect of anoxia on methemoglobinemia from aniline derivatives (i.v.)

DRUG	DOG	MAXIMAL PER CENT MHb FORMED	
		Ground level	Altitude
Nitrosobenzene	1	41.3	32.9
	2	40.0	33.1
	3	54.8	35.7
	4	53.0	43.9
	5	38.1	23.0
	6	50.8	34.3
	12	38.1	26.5
	13	48.1	27.0
	20	44.4	33.8
	21	58.8	44.8
P-aminophenol	9	24.2	7.6
	10	32.3	13.1
	11	23.0	9.8

mgm./kgm. The results of these runs are shown in table 4 in which it is seen that anoxia inhibits MHb formation by both of the aniline intermediates.

Discussion. The effect of oxygen tension on the formation of MHb was

discussed by Neill and Hastings in 1925 (5). They found that the rate of *in vitro* oxidation of Hb solutions by pneumococcus extract is the resultant of two reactions which are affected oppositely by oxygen tension. The autoxidizable substance in pneumococcus extract which oxidized Hb required a certain minimal oxygen tension to furnish adequate concentration of the oxidized form. On the other hand, a high oxygen tension, in light of the fact that reduced Hb rather than oxyhemoglobin is the form susceptible of oxidation, did not favor MHb formation *in vitro.* In the pneumococcus extract-hemoglobin system an oxygen tension of 20 mm. proved optimal. Brooks has shown similarly (6) that the autoxidation of purified Hb to MHb in solution occurs most readily at low oxygen pressures, since the concentration of the oxidizable form of hemoglobin increases with decreasing oxygen pressure. The latter consideration would be in harmony with the high MHb levels after nitrite at altitude but would appear to be incompatible with the low levels after aniline unless the oxidation of aniline or the autoxidation of the compound actually responsible for the catalytic MHb formation is greater at atmospheric oxygen tension (152 mm.) than at the tensions maintained in the anoxia experiments (49 mm.). That the autoxidation of the active intermediary of aniline may be impaired at altitude is suggested by the attenuation of nitrosobenzene activity during hypoxemia. Presumably the oxidation of aniline to the hypothetical intermediate may also be effected. It is evident the optimum oxygen tension for MHb formation by aniline must lie above that for the pneumococcus extract. Keilin and Hartree state that nitrosobenzene forms a complex with Hb *in vitro* which breaks down only in the presence of oxygen to give MHb and a reduced form of nitrosobenzene (7).

In vivo the fluctuation of oxygen partial pressure favors the oxidation of Hb by these substances by making available in venous blood high concentrations of reduced Hb and in arterial blood high enough oxygen tensions to permit oxidation of the amine group or autoxidation of the reduced form of the catalytic agent.

Individual variation in methemoglobinemia after oral but uniformity of response after i.v. administration of aniline was noted by Cox and Wendel (8). Our experiments demonstrate the greater susceptibility of one of the three dogs which were subjected to a series of injections. Since the per cent MHb was more nearly the same in these dogs after nitrite it would seem the greater and more prolonged methemoglobinemia from aniline in dog 3 must be an idiosyncrasy for aniline alone. In vitro experiments[3] with erythrocytes of the six dogs used in tables 1 and 2 have shown that the cells of all dogs develop about the same level of MHb from a standard dose of nitrite and that the rate of MHb reduction is about the same for all dogs except dog 4, which is considerably slower.

SUMMARY AND CONCLUSIONS

(1) Hypoxemia reduces the per cent MHb attained in dogs after intravenous aniline, p-aminophenol or nitrosobenzene but not that reached after i.v. nitrite.

[3] Unpublished results.

(2) Reduced atmospheric pressure inhibits MHb formation resulting from cutaneous application of xylidine in cats or aniline in dogs.

(3) Daily variation in response to intravenous aniline and individual variation in this respect have been encountered. The individual variation in reaction to aniline was not observed in response to nitrite.

REFERENCES

1. von Oettingen, W. F., Neal, P. A., Sievers, R. F., et al.: National Institute of Health Bulletin No. 188, 1947.
2. Horecker, B. L., and Brackett, F. S.: J. Biol. Chem., 152: 669, 1944.
3. Andrews, H. L., and Horecker, B. L.: Rev. Sci. Instruments, 16: 148, 1945.
4. Horecker, B. L.: J. Indust Hyg. Toxicol., 27: 85, 1945.
5. Neill, J. M., and Hastings, A. B.: J. Biol. Chem., 63: 479, 1925.
6. Brooks, J.: Proc. Roy. Soc. (London), 118: Ser. B: 560, 1935.
7. Keilin, D., and Hartree, E. F.: Nature, 151: 390, 1943.
8. Cox, W. W., and Wendel, W. B.: J. Biol. Chem., 143: 331, 1942.

EFFECT OF CONVULSANT AND ANTICONVULSANT AGENTS ON THE ACTIVITY OF CARBONIC ANHYDRASE

CLARA TORDA AND HAROLD G. WOLFF

New York Hospital and the Departments of Medicine (Neurology) and Psychiatry, Cornell University Medical College, New York, N. Y.

Received for publication December 3, 1948

The concentration of carbon dioxide (and therefore bicarbonate) available for metabolic processes in the brain may be an essential factor in the induction and prevention of convulsive seizures. Gibbs, Lennox, and Gibbs (1) have observed that the carbon dioxide content of both the arterial and the internal jugular blood is unusually high in patients with convulsive seizures. Alterations in the carbon dioxide tension of blood passing through the brain have a profound effect on the abnormal electrical activity of the brain, grand mal being accentuated by high tension of carbon dioxide.

From the biochemical point of view carbon dioxide is an essential factor in regulating many phases of tissue metabolism. One may assume that during convulsive seizures some disturbances in one or more of the processes regulated by the presence of carbon dioxide (and bicarbonate) occur.

Many metabolic processes modified by carbon dioxide (and bicarbonate) are not yet identified but the processes involved in carbon dioxide uptake and release are well studied. Carbonic anhydrase is the enzyme that accelerates the solution and evolution of carbon dioxide ($CO_2 + H_2O \rightleftharpoons H_2CO_3$). An inhibition of the activity of carbonic anhydrase may reduce the uptake of bicarbonate from brain by the blood.

In the following the effect of certain convulsant and anticonvulsant agents on the activity of carbonic anhydrase was investigated to ascertain whether these substances exert any effect on the activity of this enzyme.

METHOD. The effect of the convulsant and anticonvulsant agents on the activity of carbonic anhydrase was studied by two colorimetric methods based on the method of Brinkman (2) consisting of estimating the reaction velocity in the presence of carbonic anhydrase of either (a) the hydration of carbon dioxide or (b) the dehydration of carbonic acid. Both processes were studied since modification of the enzyme activity reflects to an equal degree on both hydration and dehydration processes and the results obtained by investigation of the two processes, if parallel, serve to control each other.

DEHYDRATION EXPERIMENTS. (Method described by Florkin, 3)—Y-shaped tubes of a total volume of 4 cc. were used. The single branch having a volume of about 1 cc. was closed and the two open parallel branches were equipped with airtight ground glass stoppers. The Y-shaped tubes were standing on their closed ends in a container filled with crushed ice. About 2 cc. of chemically pure mercury were introduced first, then 1 cc. of distilled water containing $NaHCO_3$ in a concentration of $5 \times 10^{-2}M$ was pipetted into one side branch and the following solutions into the other: (1) 1 cc. of distilled water (previously saturated with air) containing NaH_2PO_4 in concentration of $1 \times 10^{-2}M$ and phenol red in concentration of $5 \times 10^{-5}M$, (2) the convulsant and anticonvulsant agents, (3) 0.01 cc. of either (a) distilled water, or (b) a solution of carbonic anhydrase, or (c) the solution of carbonic anhydrase previously boiled and cooled. Both side branches were carefully stoppered expelling all

air space between the solutions and the stoppers by addition of some mercury, if necessary. The stoppers were held tightly in place with springs.

The tube was placed carefully into the ice and kept there for at least 10 minutes to secure cooling to 0°C. Afterwards, the tubes were inverted a few times to secure complete and rapid mixing by the mercury and were replaced into the ice. The time elapsing between this mixing and the occurrence of the color change (from yellow to pink) was noted. Usually several tubes were run in parallel containing in duplicate the following mixtures: bicarbonate, phosphate with phenol red, and either (1) carbonic anhydrase, or (2) carbonic anhydrase with a convulsant or anticonvulsant agent, or (3) boiled carbonic anhydrase, or (4) boiled carbonic anhydrase with a convulsant or anticonvulsant agent.

Controls. Mixtures without carbonic anhydrase and with boiled carbonic anhydrase served as controls.

Hydration. (Method described by Leiner, 4)—The experiments were performed as described above except that 1 cc. of distilled water containing carbon dioxide in a concentration of $0.5 \times 10^{-4} M$ was pipetted into one side branch of the Y shaped tube and the following solutions in the other: (1) 1 cc. of distilled water containing $NaHCO_3$ in a concentration of $2 \times 10^{-2} M$, Na_2CO_3 in a concentration of $2 \times 10^{-3} M$, and phenol red in a concentration of $1 \times 10^{-4} M$, (2) the convulsant and anticonvulsant agents, (3) 0.01 cc. of either (a) distilled water, or (b) carbonic anhydrase, or (c) boiled and cooled carbonic anhydrase.

Controls. Mixtures without carbonic anhydrase and with boiled carbonic anhydrase served as controls.

Convulsant and anticonvulsant agents. The substances were used in very low concentrations (1×10^{-4} to $1 \times 10^{-6} M$) to cause negligible changes in the buffering ability of the bicarbonate carbonate and bicarbonate phosphate solutions. For this reason the effect of a large number of known convulsant and anticonvulsant agents could not be tested. All substances used were tested for their effect on the reaction velocity of solution and evolution of carbon dioxide in the absence of active carbonic anhydrase. Any change in the velocity of color change due to the presence of the convulsant and anticonvulsant agents was taken in consideration by the calculation.

Preparation of carbonic anhydrase. Carbonic anhydrase was prepared following the method of Meldrum and Roughton (5). Human blood was withdrawn from the cubital vein and defibrinated by stirring with a glass rod. The red blood cells were separated by centrifugation and were washed three times with cold isotonic saline solution. Cold saline solution was used to decrease the inactivation of the enzyme by heat. To 10 cc. washed red cells 6 cc. distilled water and 4 cc. ethyl alcohol were added and the mixture was shaken at room temperature for about one minute adding 5 cc. chloroform. On centrifuging for about 10 minutes at 3500 rpm a three phasic system is formed, consisting of a top layer of enzyme solution, a central layer of denaturated protein, and a bottom layer of chloroform. The top layer may be kept for many weeks in the refrigerator without an appreciable loss of its enzymatic activity.

The enzyme solution was prepared daily from this stock solution and contained as much enzyme as was needed to complete the color changes in one minute if added to the mixtures in a volume of 0.01 cc. The reaction time in the absence of the enzyme was three minutes.

Calculation—hydration experiments. The time elapsing between mixing the solutions and the occurrence of the end point of color change from purple to yellow was measured with a stop watch. The color change can be detected with an accuracy of less than 1 second. The reaction time in the mixtures containing carbonic anhydrase without convulsant and anticonvulsant agents (on the average 60 seconds) served as 100 per cent. The reaction time in the presence of the convulsant and anticonvulsant agents was expressed as a percentage of 60 seconds. This treatment of the results is permissible because, within wide limits, the reaction time and enzyme activity are inversely proportional. All results deviating from 100 per cent by more than twice the square root of the sum of the squares of the standard error of mean of the controls and the standard error of mean of the experiments were considered significant effects ($2\sqrt{S.E.^2(\text{control}) \pm S.E.^2(\text{experiment})}$).

TABLE 1

Effect of convulsant and anticonvulsant agents on hydration of carbon dioxide by carbonic anhydrase

SUBSTANCE	REACTION TIME OF MIXTURES CONTAINING THE SUBSTANCES EXPRESSED IN PER CENT OF THE REACTION TIME OF MIXTURES WITHOUT THE SUBSTANCES (FOLLOWED BY THE STANDARD ERROR OF MEAN).* Concentration of the substances in mols.		
	10^{-4}	10^{-5}	10^{-6}
Acetylcholine	154 ± 1.4	140 ± 1.9	133 ± 1.2
Camphor	132 ± 1.2	119 ± 1.1	111 ± 0.5
Dichlorodiphenyltrichloroethane	—	138 ± 1.3	118 ± 0.9
Digitoxin	—	124 ± 1.4	154 ± 1.5
Pentamethylene tetrazol	140 ± 1.0	—	—
Picrotoxin	146 ± 2.0	—	—
Scilliroside	147 ± 2.2	137 ± 1.5	113 ± 1.0
Strychnine	120 ± 1.7	120 ± 0.4	100 ± 0.3
Ouabain	113 ± 1.2	140 ± 1.0	—
Hydantoin	55 ± 1.3	81 ± 0.9	—
Methyl-phenyl-ethyl hydantoin	72 ± 1.8	80 ± 1.1	—
Phenobarbital	50 ± 1.5	68 ± 1.7	—

* Each value represents the average of ten separate experiments.

TABLE 2

Effect of convulsant and anticonvulsant agents on dehydration of carbonic acid by carbonic anhydrase

SUBSTANCE	REACTION TIME OF MIXTURES CONTAINING THE SUBSTANCES EXPRESSED IN PER CENT OF THE REACTION TIME OF MIXTURES WITHOUT THE SUBSTANCES (TEN EXPERIMENTS FOR EACH GROUP) Concentration of the substances in mols.		
	10^{-4}	10^{-5}	10^{-6}
Acetylcholine	150	140	130
Camphor	150	120	—
Dichlorodiphenyltrichloroethane	—	140	—
Digitoxin	—	125	150
Pentamethylene tetrazol	150	130	—
Picrotoxin	140	125	—
Scilliroside	160	150	—
Strychnine	120	120	—
Ouabain	120	150	—
Hydantoin	50	70	—
Methyl-phenyl-ethyl hydantoin	60	60	—
Phenobarbital	60	70	—

Dehydration experiments. Since the buffer combination used permits a gradual change of the color from yellow to pink, detection of the endpoint of the reaction is very difficult with an accuracy of less than 10 per cent. For this reason the results were not treated statistically.

RESULTS. The activity of carbonic anhydrase was inhibited in the presence of the convulsant agents used (acetylcholine, camphor, dichlorodiphenyl trichloroethane, digitoxin, pentamethylene tetrazol, picrotoxin, scilliroside, strychnine, and ouabain) in concentrations of 1×10^{-4} M and less, the greatest inhibition being, on the average, 54 per cent (see tables 1 and 2).

The activity of carbonic anhydrase increased in the presence of the anticonvulsant agents used (hydantoin, methyl-phenyl-ethyl hydantoin, and phenobarbital) in concentrations of 1×10^{-4} M and less, the greatest increase of activity being, on the average, 50 per cent (see tables 1 and 2).

DISCUSSION. Nerve activity under an adequate supply of oxygen does not parallel the activity of carbonic anhydrase. Membrane and spike potentials of animal nerve (6) and human encephalogram (7) in the presence of sulfanilamide, a specific inhibitor of carbonic anhydrase (8), resemble those of objects with uninhibited carbonic anhydrase. Under anoxic conditions, however, membrane and spike potentials decrease if carbonic anhydrase is inhibited (6). Therefore, at least under anoxic conditions, there are indications that carbonic anhydrase contributes to the maintenance of optimal nerve function.

The results presented in this investigation suggest that convulsant agents, by inhibiting the activity of carbonic anhydrase (from blood and probably from brain), decrease the removal of carbon dioxide and bicarbonates from the brain. This mechanism may be one of the means by which convulsant agents induce their effect. Furthermore, anticonvulsant agents, by increasing the activity of carbonic anhydrase (from blood and probably from brain), increase the removal of carbon dioxide and bicarbonates from the brain.

SUMMARY

1. The effect of certain convulsant and anticonvulsant agents on the activity of carbonic anhydrase was investigated.

2. The activity of carbonic anhydrase was inhibited by convulsant agents in low concentrations.

3. The activity of carbonic anhydrase was increased by anticonvulsant agents in low concentrations.

The authors wish to express their gratitude to Dr. C. R. Noller for the generous supply of the scilliroside and to Sandoz Chemical Works, Inc., for the methyl-phenyl-ethyl hydantoin.

REFERENCES

1. GIBBS, E. L., LENNOX, W. G., AND GIBBS, F. A.: Arch. Neurol. and Psychiat., 43: 223, 1940.
2. BRINKMAN, R.: J. Physiol., 40: 283, 1934.
3. FLORKIN, M.: Archiv. internat. Physiol., 40: 283, 1934.
4. LEINER, M.: Zeitschr. f. vergl. Physiol., 26: 416, 1938.
5. MELDRUM, N. U., AND ROUGHTON, F. J.: J. Physiol., 80: 113, 1934.
6. SHANES, A. M.: Fed. Proc., 5: 93, 1946.
7. DAVENPORT, H. W.: J. Neurophysiol., 9: 41, 1946.
8. MANN, T., AND KEILIN, D.: Nature, 146: 164, 1940.

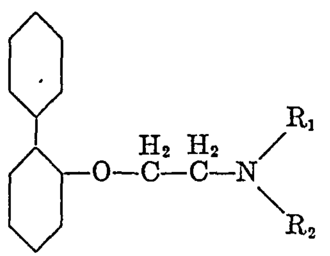
ADRENERGIC BLOCKING DRUGS. IV. ANTAGONISM OF EPINEPHRINE AND HISTAMINE WITH 2-(2-BIPHENYLOXY)-2′-CHLORODIETHYLAMINE DERIVATIVES[1]

EARL R. LOEW[2] AND AUDREY MICETICH

Department of Pharmacology, University of Illinois, College of Medicine, Chicago 12

Received for publication December 9, 1948

Following the discovery of the adrenergic blocking action of Dibenamine (N-(2-chloroethyl)dibenzylamine·HCl) by Nickerson and Goodman (1, 2) it was found that other 2-halogenated ethylamines exerted similar action or exerted both antihistamine and adrenergic blocking action (3–14), as with the 1-naphthalenemethylamines (10). The present article concerns the antihistamine and adrenergic blocking action, as well as the acute toxicity, of a series of 2-(2-biphenylyloxy)-2′-chlorodiethylamines (table 1) which are characterized by the following structural formula:



R_1—alkyl
R_2—2—chloroalkyl or alkyl

Aqueous solutions of the hydrochloride salts were freshly prepared for use in daily experiments since compounds of this type are known to be unstable, especially if heated or in alkaline solution. These various alkyl homologues were all readily soluble in water to the extent of 1.0 per cent or more.

Several types of tests were applied to detect pharmacological activity and partially to evaluate the activity in relation to acute toxicity. Initially, evidence of the ability of compounds to block epinephrine was accumulated by determining which compounds diminished the toxicity of epinephrine in mice, and then the majority of compounds were tested for antihistamine action as indicated by their ability to diminish the severity of histamine-induced bronchiospasm in guinea pigs. The acute oral toxicity was then determined in mice. Finally, further information relating to antihistamine, adrenergic blocking and atropine-like action was gained by ascertaining whether intravenous injections in anesthetized dogs effected diminution or blocking of the pressor responses to epinephrine or of the depressor responses to small doses of histamine and acetylcholine.

RESULTS

A. *Acute oral toxicity and reduction of epinephrine toxicity in mice.* The ability of compounds to diminish the toxicity of epinephrine injected in mice con-

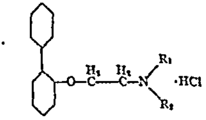
[1] We gratefully acknowledge the assistance of Parke, Davis and Company for financial support and the compounds which were synthesized by Drs. G. Rieveschl, Jr., and R. W. Fleming.

[2] Present address: Department of Physiology, Boston University School of Medicine, 80 East Concord Street, Boston 18, Mass.

stituted presumptive evidence of adrenergic blocking action. Details regarding this screening procedure (9) as well as its application in determining the activity of a series of 1-naphthalenemethylamines (10) have been published. Essentially the method consisted of administering orally two or more doses of each compound to groups of twenty mice which were injected intraperitoneally with epinephrine hydrochloride two hours later. In forty control groups of twenty mice each

TABLE 1

The antagonism of epinephrine and histamine



Comp. No.*	R_1	R_2	Reduction of epinephrine toxicity in mice effective oral dose† ± S. E.	Acute toxicity oral, mice LD_{50} ± S. E.	Ratio of oral LD_{50} to oral dose effective against epinephrine	Diminution of histamine-induced bronchiospasm in guinea pigs M. E. D., subc.
			mgm./kgm.	*mgm./kgm.*		*mgm./kgm.*
1	Methyl	2-chloroethyl	6.0 ± 1.1	903 ± 53	150	1.5
2 (SY-8)	Ethyl	2-chloroethyl	5.0 ± 0.6	759 ± 53	151	6.0
3	n-Propyl	2-chloroethyl	3.1 ± 0.6	944 ± 48	304	12.5
4	Isopropyl	2-chloroethyl	4.8 ± 0.6	922 ± 71	192	>12.5
5 (SY-30)	n-Butyl	2-chloroethyl	3.0 ± 0.3	749 ± 36	249	>25.0
6	Sec.-butyl	2-chloroethyl	5.1 ± 0.5			
7	Isobutyl	2-chloroethyl	4.5 ± 0.3	1159 ± 72	257	
8	n-Amyl	2-chloroethyl	2.6 ± 0.3	1060 ± 42	407	>12.5
9 (SY-74)	n-Hexyl	2-chloroethyl	3.4 ± 0.6	959 ± 78	282	>25.0
10	Allyl	2-chloroethyl	2.5 ± 0.4	955 ± 38	382	>12.5
11	Ethyl	2-chloropropyl	21.0 ± 5.3			12.5
12	Ethyl	Ethyl	Ineffective at 50			
13	2- (3-Biphenylyloxy) -2'-chlorotriethylamine		"			
14	2-(2-Cyclohexylphenoxy)-2'-chlorotriethylamine		"			>25.0
15	3- (4-Biphenylyloxy)-N-(2-chloroethyl)-N-ethyl -propylamine		"			

* The hydrochloride salt was used in all instances.

† The effective oral dose represents the calculated amount required to reduce mortality of mice from 67.0 to 33.5 per cent following intraperitoneal injection of a standard dose of epinephrine (cf. ref. 9 for details).

treated with saline and injected with epinephrine the mortality was 67.0 ± 1.53 per cent. With each test compound, determination was made of the dose which would protect one-half of the mice, i.e., reduce mortality from 67.0 to 33.5 per cent.

Examination of the doses required to reduce epinephrine toxicity (table 1) indicates that approximately the same degree of activity was exerted by all the alkyl homologues (nos. 1 to 10) which possessed a 2-chloroethyl group. A

remarkable degree of activity is indicated by the fact that small oral doses ranging from 2.5 to 6.0 mgm./kgm. were effective. Modifications in chemical structure which lessened activity are comparable to those found in the study of derivatives of Dibenamine (15) and 1-naphthalenemethylamine derivatives (10). Addition of a single carbon atom in the chlorinated alkyl group greatly reduced activity (compare nos. 11 and 2) and absence of the chlorine atom caused loss of activity (no. 12). Furthermore, no activity was demonstrated with compounds in which the side chain was in the 3-position of the biphenyl group (no. 13) or after saturation of one phenyl ring (no. 14).

With each of the most active compounds the small magnitude of the oral effective dose is in sharp contrast to the large oral LD_{50} determined also in mice as previously described (10). The appreciable ratio between LD_{50} and the effective dose (table 1) indicates a good factor of safety, at least in mice, and since the LD_{50} always exceeded 750 mgm./kgm. the acute toxicity data would not eliminate any compound from further consideration as a possible effective adrenergic blocking drug with low toxicity.

It is of interest that these adrenergic blocking compounds as well as the others we have investigated, not only reduced mortality following epinephrine injection but markedly alleviated symptoms. In treated mice the fur remained sleek in appearance following epinephrine injection and did not become erect to give the mice a noticeable rounded contour. Motor activity remained nearly normal and mice seldom assumed a sprawling position as when definitely intoxicated with epinephrine. Rapid and dyspneic breathing were less noticeable. It should be mentioned that no bloody, frothy exudate appeared at the nostrils even in the mice receiving only epinephrine, which is in contrast to the effects of epinephrine noted in rabbits (16, 17). Adrenergic blocking drugs also diminished the ability of epinephrine to cause exophthalmia and opacity of the cornea. Thus, adrenergic blocking drugs prevented almost all of the usual changes in behavior and appearance of mice injected with toxic doses of epinephrine.

When large doses of the biphenylyloxyethyl derivatives were administered orally to mice, no definite evidence of hyperexcitability, tremors or convulsions occurred. Ataxia and depression were apparent and dyspnea, as well as asphyxial type of convulsive movements, were usually observed in terminal stages. Deaths were most common within one or two hours, with only a few deaths occurring thereafter up to a total of five days. The low incidence of late deaths lessens the possibility that death is commonly closely related to complete and prolonged blocking of adrenergic effector organs which might cause death by interfering with temperature regulation or other functions partially controlled by the sympathetic nervous system.

B. *Diminution of histamine-induced bronchiospasm in guinea pigs.* Representative compounds from the series under investigation were injected subcutaneously in groups of twelve guinea pigs 30 minutes before subjecting the animals to an histamine aerosol which killed 92 per cent of those in a control group of twelve animals. Effectiveness of compounds in reducing bronchioconstriction and asphyxial death is indicated by the magnitude of the approximate minimal effective doses (table 1) required to reduce mortality.

Antihistamine action was greatest with the methyl homologue (no. 1) which was effective in a dose of 1.5 mgm./kgm. Activity decreased progressively as methyl groups were added to the alkyl chain (nos. 2 to 9) and was decreased by substitution of the 2-chloropropyl for the 2-chloroethyl group (compare nos. 11 and 2). None of these compounds exerted an antihistamine action comparable in degree to that of diphenhydramine hydrochloride (Benadryl), pyranisamine (Neoantergan), tripelennamine (Pyribenzamine) and other antihistamine drugs which are employed clinically. Since lengthening of the alkyl chain progressively decreased antihistamine action, it is apparent that such action was not proportional to the epinephrine antagonism demonstrated in mice which appeared to be undiminished by lengthening of the alkyl chain.

C. *Epinephrine and histamine antagonism in dogs.* The majority of compounds were injected into one or more dogs anesthetized with pentobarbital sodium under conditions which permitted a study of blood pressure responses for detection of epinephrine blocking or reversal, antagonism of the depressor action of histamine and evidence of atropine-like action as indicated by diminished depressor responses to acetylcholine. These experiments were conducted in a manner identical to that employed during the study of 1-naphthalenemethylamines (10). Two intravenous injections of epinephrine hydrochloride (10 microgm.), acetylcholine bromide (25 microgm.) and histamine diphosphate (50 microgm.) were made prior to slow intravenous injection (2 minutes) of a test compound. Beginning 10, 60 and 120 minutes after injection of the test compound, injections of each of the pressor and depressor drugs were repeated twice. Table 2 contains the mean of the several averaged paired responses occurring before treatment and at the 10 to 30 and 120 to 150 minute periods after treatment, and the differences are indicative of the degree of antagonism which occurred with each of three representative alkyl homologues.

In doses of 3.0 mgm./kgm., intravenously, the ethyl (no. 2 or SY-8), n-butyl (no. 5 or SY-30) and n-hexyl (no. 9 or SY-74) all blocked the pressor responses to injected epinephrine; reversal frequently occurred as indicated by the fact that the mean differences recorded (table 2) are greater than the control pressor responses.

In one-half or three-fourths of the dogs treated with each compound there was an immediate, small, fleeting, pressor response to epinephrine which was usually followed by a hypotension. These pressor spikes were not considered in measuring and recording the hypotensive responses (table 2), except when no reversal occurred, in which case the pressor response was considered as a diminished pressor response to epinephrine. It is probable that the initial pressor spike is caused by epinephrine acting on the heart and causing an increased cardiac output which is not diminished by reasonable doses of known adrenergic blocking agents (2, 10). Epinephrine reversal occurred within 10 minutes and, as the data reveal, was undiminished after 150 minutes when the experiments were terminated. Thus, epinephrine antagonism was rapid in onset and of long duration. The data do not suggest that the higher alkyl homologues (nos. 5 and 7) were any more effective than the ethyl compound (no. 2) and thus neither dog nor mice experiments revealed significant differences in potency.

TABLE 2

Effect of three 2-(2-biphenylyloxy)-2'-chlorodiethylamines on pressor responses to epinephrine and depressor responses to histamine and acetylcholine in dogs

COMP. NO.	DOSE I.V.	NO. EXPTS.	EPINEPHRINE HYDROCHLORIDE 10 MICROGM.				
			Control mean pressor response	After treatment, mean difference ± S. E.*			
				10–30 min.	P	120–150 min.†	P
	mgm./kgm.		mm. Hg	mm. Hg		mm. Hg	
2 SY-8 ethyl	3.0	4	35.5	40.1 ± 10.2	0.03	54.5 ± 9.6	0.01
5 SY-30 n-butyl	3.0	5	24.6	34.2 ± 7.1	0.01	29.4 ± 5.1	<0.01
9 SY-74 n-hexyl	3.0	4	36.0	47.3 ± 2.8	0.01	46.2 ± 2.8	<0.01
			HISTAMINE DIPHOSPHATE 50 MICROGM.				
			Control mean depressor response	After treatment, mean difference ± S. E.‡			
				10–30 min.	P	120–150 min.†	P
			mm. Hg	mm. Hg		mm. Hg	
2 SY-8 ethyl	3.0	4	37.9	30.4 ± 5.5	0.01	18.4 ± 5.0	0.04
5 SY-30 n-butyl	3.0	5	38.9	26.8 ± 7.0	0.02	23.5 ± 6.6	0.02
9 SY-74 n-hexyl	3.0	4	44.2	25.1 ± 4.4	0.01	16.0 ± 5.7	0.07
			ACETYLCHOLINE BROMIDE 25 MICROGM.				
			Control mean depressor response	After treatment, mean difference ± S. E.‡			
				10–30 min.	P	120–150 min.†	P
			mm. Hg	mm. Hg		mm. Hg	
2 SY-8 ethyl	3.0	4	35.0	14.1 ± 6.5	0.1	11.6 ± 8.3	0.3
5 SY-30 n-butyl	3.0	5	35.8	8.02 ± 3.0	0.07	11.9 ± 5.0	0.1
9 SY-74 n-hexyl	3.0	4	38.1	7.20 ± 8.2	0.4	11.2 ± 5.8	0.15

* Mean differences greater than the control pressor responses indicate epinephrine reversal (depressor response), and those which approximately equal control responses indicate marked diminution or blocking. After treatment with each of the three drugs, epinephrine usually elicited a pressor spike ranging from 5 to 30 mm. Hg which preceded the depressor response.

† Data related to responses at the 60 to 90 minute interval are omitted since they were nearly identical to those recorded for the 120 to 150 minute interval.

‡ Depressor responses to histamine and acetylcholine were not blocked as indicated by mean differences which never equal the control values.

Likewise, each of the three alkyl homologues diminished the depressor responses to histamine a comparable degree, even though the n-butyl and n-hexyl compounds (nos. 5 and 9) had failed to diminish histamine-induced bronchioconstriction in guinea pigs following doses of 25.0 mgm./kgm., subcutaneously. The wide differences in antihistamine potency based on reactions of bronchiolar muscle in guinea pigs were not apparent from the altered depressor responses to histamine in dogs. Possibly the vascular reactions do not readily reveal differences in potency or the various alkyl homologues have a selective antihistamine action on some vascular components which is similar for the various compounds. Interpretation is difficult because of differences in animal species and routes of administration. The data suggest that the antagonism of the depressor effects of histamine was somewhat decreased 120 to 150 minutes after treatment. The compounds usually failed to significantly reduce the mean depressor responses to acetylcholine and since reduced responses were inconstant and not of great magnitude it is concluded that no significant degree of atropine-like action was demonstrated.

One or more identical experiments were made in dogs with most of the remaining compounds in the series. Comparable results were obtained with all the alkyl homologues which contained a 2-chloroethyl group. No epinephrine or histamine antagonism or atropine-like action was exerted when the chlorine atom was substituted with hydrogen (no. 12) and no activity was exhibited following alterations in or on the biphenyl nucleus (nos. 13, 14, 15). In summary, the results indicate that the mouse screening method revealed adrenergic blocking activity which was confirmed in dogs, and antihistamine action was indicated by responses of bronchioles in guinea pigs and depressor responses in dogs. The 2-chloroethyl group was essential for maximal adrenergic blocking and histamine antagonism.

Discussion. Adrenergic blocking action proved to be the most prominent pharmacological action of 2-(2-biphenylyloxy)-2′-chlorodiethylamine derivatives in which the tertiary nitrogen atom contained a 2-chloroethyl group and an alkyl group. A weak antagonism of histamine was exerted by the lower alkyl homologues (methyl, ethyl and n-propyl) as indicated by diminished histamine-induced bronchiospasm in guinea pigs. These compounds as well as the higher alkyl homologues (n-butyl and n-hexyl; table 2) appeared to exert appreciable diminution in the ability of histamine to effect depressor responses in dogs. The results suggested that antihistamine action was more easily elicited on the vascular system of dogs than on bronchiolar smooth muscle of guinea pigs. None of the compounds decreased the depressor responses to acetylcholine sufficiently to establish a significant degree of atropine-like action.

Qualitatively, these 2-(2-biphenylyloxy)-2′-chlorodiethylamines are comparable to the 1-naphthalenemethylamines recently described (8–11) although some compounds in the latter series proved much more potent with respect to both adrenergic blocking and antihistamine action. These 2-halogenated ethylamines which exert dual antagonism of epinephrine and histamine differ from related compounds, Dibenamine (1, 2, 15, 18) and benzhydrylamines (3,

9), which are less potent with respect to adrenergic blocking action and exert no important degree of histamine antagonism. In comparison with these compounds Dibenamine is unique by virtue of its slow onset of action. Dibenamine and the 2-halogenated ethylamines which we have investigated have the common property of long duration of action.

More evidence concerning adrenergic blocking action was adduced in the experiments with N-[2-(2-biphenylyloxy)ethyl]-N-(2-chloroethyl)butylamine·HCl (SY-30 or no. 5 in tables 1 and 2) which induced epinephrine reversal in dogs, blocked or reversed pressor responses to anoxia and to nicotine injections, and significantly diminished pressor responses to carotid occlusion (8, 11).

SUMMARY

2-(2-Biphenylyloxy)-2′-chlorodiethylamines with N-substituted alkyl and 2-chloroethyl groups exerted a moderate degree of adrenergic blocking action in mice and dogs and diminished the depressor action of histamine in dogs. The lower alkyl homologues weakly antagonized the bronchioconstrictive action of histamine in guinea pigs. In dogs, depressor responses to acetylcholine were not significantly diminished. The properties of these compounds are briefly compared and contrasted to those possessed by other 2-halogenated ethylamines.

REFERENCES

1. Nickerson, M., and Goodman, L. S.: Fed. Proc., 5: 194, 1946.
2. Nickerson, M., and Goodman, L. S.: This Journal, 89: 167, 1947.
3. Loew, E. R., and Micetich, A.: Fed. Proc., 6: 351, 1947.
4. Loew, E. R., Micetich, A., and Achenbach, P.: Fed. Proc., 6: 351, 1947.
5. Achenbach, P., and Loew, E. R.: Fed. Proc., 6: 304, 1947.
6. Loew, E. R.: Physiol. Rev., 27: 542, 1947.
7. Loew, E. R.: Ann. N. Y. Acad. Sci., (in press), 1948.
8. Stone, C., Achenbach, P., and Loew, E. R.: Fed. Proc., 7: 258, 1948.
9. Loew, E. R., and Micetich, A.: This Journal, 93: 434, 1948.
10. Loew, E. R., and Micetich, A.: This Journal, 94: 339, 1948.
11. Stone, C., and Loew, E. R.: This Journal, 94: 350, 1948.
12. Wells, J. A., and Rall, D. P.: Fed. Proc., 7: 264, 1948.
13. Hunt, C. C.: Fed. Proc., 7: 229, 1948.
14. DeVleeschhouwer, G. R.: Proc. Soc. Exper. Biol. and Med., 67: 298, 1948.
15. Nickerson, M., Nomaguchi, G., and Goodman, L. S.: Fed. Proc., 5: 195, 1946.
16. Luisada, A.: Arch. f. exper. Path. u. Pharmakol., 132: 313, 1928.
17. Halpern, B. N., Hamburger, J., and Cruchaud, S.: Acta Allergologica, 1: 97, 1948.
18. Nickerson, M., and Nomaguchi, G.: This Journal, 93: 40, 1948.

A STUDY ON MESCALINE IN HUMAN SUBJECTS[1]

KURT SALOMON,[2] BEVERLY WESCOTT GABRIO,[2] AND THOMAS THALE

Biochemical Research Laboratory of the Departments of Neuropsychiatry and Radiology, Washington University, St. Louis, Missouri

Received for publication December 13, 1948

Mescaline, 3,4,5,trimethoxyphenylethylamine, produces hallucinations in the visual modality. This phenomenon has been extensively investigated from a psychiatric and a psychological point of view, but only very few studies of the chemical fate of mescaline in man are available, while to our knowledge, there are no data concerning the physiology of the visual mechanism in response to mescaline.

The hallucinations produced by this drug are due to a great extent to the presence of methoxyl groups in the mescaline molecule, since beta-phenylethylamine, a homologous substance without methoxyl groups, does not cause hallucinations. However, the methoxyl groups alone are not sufficient to produce hallucinations since Slotta and Müller (1) showed that trimethoxyphenylacetic acid, the *in vitro* oxidation product of mescaline, does not produce hallucinatory phenomena in man. It is evident, therefore, that the amino group in the side chain also plays an essential part.

Slotta and Müller were able to isolate from the urine of mescaline-fed humans a substance which contained one methoxyl group only, thus indicating that some of the mescaline ingested is metabolized. Their experiments revealed also that trimethoxyphenylacetic acid is not a step in the *in vivo* decomposition process. They did not investigate whether or not mescaline was excreted unchanged in the urine. However, Richter (2) showed that after oral ingestion of mescaline hydrochloride (191 mgm. base), 58 per cent was excreted unchanged in the urine about 18 hours after ingestion. This observation is in agreement with the findings of Bernheim and Bernheim (3), and Blaschko (4) that the presence of methoxyl groups renders the mescaline molecule more resistant to oxidation.

In this investigation of mescalinized human subjects, we were concerned primarily with two points: 1. A quantitative estimation of the urinary excretion of the methoxyl groups of mescaline. 2. Color perception before and after medication to determine if changes in this physiological function are correlated with visual hallucinations.

EXPERIMENTAL. Six subjects were used in this investigation. The following doses were given: 200 mgm. mescaline sulfate[3] (137.8 mgm. base) to one schizophrenic female; 300 mgm.

[1] This investigation was supported in part by funds of the Frank Phillips Foundation, and the Simon Grant for Research. Presented in part at the annual meeting of the Federation of American Societies for Experimental Biology, Chicago, Illinois, May 19–23, 1947.

[2] Present address: University of Rochester School of Medicine and Dentistry, Rochester, New York.

[3] The mescaline sulfate was generously supplied by the Hoffmann-La Roche Company.

mescaline sulfate (206.7 mgm. base) to one schizophrenic male and one schizophrenic female; 400 mgm. mescaline sulfate (275.6 mgm. base) to two schizophrenic males and one neurotic male. All doses were given orally in the morning while the subjects were in a fasting state. Detailed information concerning the subjects used, the hallucinatory responses, and changes in visual imagery, will be published elsewhere.

1. *Urinary excretion of methoxyl groups.* Urine specimens were collected prior to medication and at stated intervals for 18 hours (2, 4, 6, 10, 14, 18) thereafter. A 40 cc. aliquot from each urine sample was extracted for mescaline by first adjusting the pH to about 9 with KOH and then extracting twice with an equal volume of a 1:1 mixture of toluene and isobutylalcohol. Preliminary experiments had shown that this mixture was superior for the extraction of mescaline. The extract was dried with anhydrous sodium sulfate. An appropriate aliquot of the dried extract was used for the determination of methoxyl groups following a modification of the Zeisel method (5). The validity of the forementioned method was tested for known amounts of mescaline added to urine samples. The error of recovery was plus or minus 4 per cent. Methoxyl groups were never found in urines of nonmedicated subjects.

After the content of methoxyl groups of aliquots of urines was determined, the remainder of all the urine samples of one particular patient was combined and extracted after alkalinization with the toluene-isobutylalcohol mixture. These extracts were used for the identification of mescaline and the investigation of the presence of possible breakdown products. Mescaline was isolated and identified as the picrate by the following method. The solvent (toluene-isobutylalcohol) was evaporated *in vacuo*. The brown residue thus obtained was treated with hot water, and the undissolved material was filtered off. The latter was dried *in vacuo* and then developed a resin-like consistency. From the filtrate, crystalline mescaline picrate was obtained and purified by repeatedly dissolving in acetone and precipitating with petroleum ether. Mescaline picrate thus obtained melted at 217–220° C. uncorrected. The mixed melting point with an authentic sample of synthesized mescaline picrate showed no depression.

A sample of the resin-like material mentioned above was analyzed for the presence of methoxyl groups. The Zeisel test revealed that the material contained methoxyl groups. In addition a qualitative test for methoxyl and other alkoxyl groups devised by Tobie (6) was applied to the resin-like material and gave a positive result. Identification of the methoxyl-containing substance or substances present in the resin-like material was not attempted.

2. *Color perception.* Color perception was tested within two days prior to the administration of the drug and again 30 to 180 minutes after the drug was given.

The apparatus used was devised by H. B. Molholm[4] who will describe it in a forthcoming publication. It is based on the principle that when lights of different intensity are seen in rapid alternation, the effect is one of flicker. When the intensity of the two sources is nearly equivalent, the flicker disappears, and the subject perceives one steady light. In this device a single source was used to produce a white light of constant intensity, and a second beam alternated with it. The second beam was modified by passage through a variable density Eastman film, manipulated by the subject. In addition, the second beam could be presented untinted or could be passed through a color filter. The following filters were used: nile green, sextant green, emerald green, lighthouse red, red-yellow, and blue.

The variable light was always presented in greater intensity than the fixed. The subject was instructed to turn a dial which increased the density of the film. When he signified that the light no longer flickered, he was told to reverse the movement and find the point at which flicker was barely apparent. This was used as the end point. The threshold for each color was determined three to five times at each testing and the mean value was used, numbers being read from a scale fixed to the variable film. The threshold for white was

---

[4] We wish to thank Dr. Molholm for making this apparatus available to us for this investigation.

determined before and after each battery of color tests. All color determinations were corrected for any shift in the threshold for white, the latter accounting for changes in the illumination, the speed of the motor which alternated lights, and the ability to perceive flicker as distinguished from color sensitivity.

RESULTS AND COMMENTS. 1. *Urinary excretion of methoxyl groups.* The various data obtained in this investigation are shown in table I. The majority of the methoxyl groups determined are considered to be representative of mescaline excreted unchanged in the urine. However, the resin-like material

TABLE I

Urinary excretion of methoxyl-containing compounds

SUBJECT	SEX	WT.	DOSE		VOLUME URINE IN 18 HOURS	TOTAL METHOXYL EXCRETED IN 18 HOURS	METHOXYL EXCRETED TO METHOXYL INGESTED
			Mescaline	Methoxyl			
		kgm.	*mgm.*	*mgm.*	*cc.*	*mgm.*	*per cent*
E†.....	M	93.6	275.6	118.5	990	43.2	36.4
P*....	M	63.0	275.6	118.5	975	31.0	26.1
J*......	M	70.5	275.6	118.5	1520	16.3	13.7
C*.....	M	55.5	206.7	88.9	2195	34.7	38.9
S*......	F	43.4	206.7	88.9	690	14.5	16.2
A*.....	F	63.2	137.8	59.3	665	5.1	8.6

* Schizophrenic.
† Neurotic.

TABLE II

Change in color perception after mescaline ingestion

SUBJECT	DOSE MESCALINE	EMERALD GREEN	NILE GREEN	SEXTANT GREEN	RED-YELLOW	LIGHTHOUSE RED	BLUE
	mgm.						
E............	275.6	−4	−1	−10	−2	−5	−15
P............	275.6	39	−6	1	0	0	4
J............	275.6	−17	−5	0	−14	13	6
C............	206.7	−15	−14	−12	−8	−24	−22
S............	206.7	−3	−10	−4	−9	−22	−6
A............	137.8	−6	−9	−7	−1	−16	−2

which remained after water extraction of the residue of the combination of all the toluene-isobutylalcohol extracts of all the subjects contained less than 10 per cent of the total methoxyl groups extracted. It might be assumed that this resin-like material contained one or a mixture of the breakdown products of mescaline. Therefore, no conclusions may be drawn concerning the type of molecule to which these methoxyl groups in the resin-like material are attached. Essentially this finding is in accordance with that of Slotta and Müller (1) who claimed that not all of the mescaline ingested leaves the body unchanged.

Although only a small percentage of the methoxyl groups excreted belonged to breakdown products of mescaline according to our experiment, we have pre-

ferred to express the excretion of the drug in table I in terms of methoxyl groups. The percentage of total methoxyl groups excreted in 18 hours to total methoxyl groups ingested varied among the subjects between 8.6 and 38.9 per cent. These values are lower than the value reported by Richter of 58 per cent, but while the excretion curve of Richter's subject showed that at 18 hours after medication mescaline excretion had approached the zero line, similar excretion curves of our subjects indicated that the mescaline had not all been excreted in this period of time. In addition, the quantitative difference in these two sets of data might be explained by the fact that we determined methoxyl groups of the urinary extract whereas Richter measured the amino groups present.

The average peak excretion of mescaline of our subjects at six hours post medication is in agreement with the maximum excretion time of Richter's subject. It is worth mentioning at this point that the most intense hallucinatory response of each subject as reported to the observers occurred in each case before the peak urinary excretion of methoxyl groups.

Further experimentation along this line with a series of normal subjects as well as schizophrenic subjects may explain from a metabolic point of view the difference mentioned by Slotta and Müller between the hallucinatory response to mescaline of normal and schizophrenic subjects.

2. *Color perception.* Table II shows the corrected changes in scores for color perception after mescaline ingestion. A negative value indicates a decreased color perception following the drug. The general trend is toward a decrease in perception. One of the schizophrenics showed a marked increase for emerald green, and a slight increase in blue, while another schizophrenic who also received 275.6 mgm. mescaline showed a slight increase in blue. The decreases in perception were greater for those receiving the smaller doses.

All subjects were ranked according to the following variables: dose, vividness of hallucinations, amount of methoxyl groups excreted, average change in color perception, and change of each individual color. Thus the subject having the greatest excretion would receive a rank of 1 for that variable, the subject excreting the next greatest amount a rank of 2, etc. Each of these variables was correlated with each other by means of the rank order method.

The following correlations were found to be significant at the 5 per cent level:

Vividness of hallucinations—Change in color perception	−.94
Vividness of hallucinations—Nile green	−.89
Change in color perception—Nile green	+.94

Sizable, but not statistically significant correlations suggested that the larger doses were associated with more vivid hallucinations, greater absolute and relative excretion of methoxyl groups and, to a lesser degree, with total amount of urine excreted. The initial suppression of urine was compensated for by a greater flow in the latter hours of observation. On the other hand, color perception, particularly for lighthouse red and nile green, was more impaired by small doses than by large ones.

Vividness of hallucinations was definitely not associated with impairment of

color perception as is shown by the large negative correlations with average change in color perception and with nile green.

The correlations involving average color change and the individual colors suggest that the effect is manifested throughout the entire range rather than in any particular range.

SUMMARY

The urinary excretion of methoxyl groups was followed quantitatively over an 18-hour period in 5 schizophrenic subjects and 1 neurotic subject after the ingestion of mescaline sulfate. Mescaline was identified in the urinary extracts, and the majority of methoxyl groups determined may be considered to be present as mescaline. Methoxyl groups belonging to breakdown products of mescaline were found in a resin-like residue of the urinary extract.

Orally administered mescaline sulfate (200–400 mgm.) produced visual hallucinations and impairment of color perception. Large doses were associated with greater excretion and more vivid hallucinations than were small doses. The greatest impairment of color vision was observed in those subjects receiving smaller doses.

We wish to thank Dr. Edwin F. Gildea, Dept. of Neuropsychiatry, for his -nterest *in this investigation.*

REFERENCES

1. Slotta, K. H., and Müller, J.: Ztschr. f. Physiol. Chem., 237-238: 14: 1936.
2. Richter, D.: Biochem. J., 32: 1763, 1938.
3. Bernheim, F., and Bernheim, M. W. C.: J. Biol. Chem., 123: 317, 1938.
4. Blaschko, H.: J. Physiol., 103: 13P, 1944.
5. J. Assoc. Official Agric. Chem., 22: 100, 1939.
6. Tobie, W. C.: Ind. Eng. Chem. (Anal. Ed.), 15: 433, 1943.

THE ABSORPTION OF PHENYLMERCURIC ACETATE FROM THE VAGINAL TRACT OF THE RAT

EDWIN P. LAUG AND FRIEDA M. KUNZE

Division of Pharmacology, Food & Drug Administration, Federal Security Agency, Washington, D. C.

Received for publication December 13, 1948

The use of phenylmercuric acetate as a spermaticide has raised the question of how much mercury may be absorbed from the vaginal tract. From the results of clinical studies Eastman and Scott (1) concluded that approximately 3 to 4 per cent of the mercury instilled into the human vagina was recoverable in a 24-hour catheter specimen of urine immediately following repeated exposure. These investigators assumed that this amount represented the total absorption, but were unable by this means to assess the amount of mercury that might have been absorbed and stored in the tissues. In the experiments to be reported it will be shown that the absorption of mercury, as reflected by its storage rather than by its excretion, can be far greater.

The principle applied in these experiments on the rat depends on the storage of mercury chiefly in the kidney, and liver (2) as a measure of absorption. It is, of course, obvious that the mercury lodging in these tissues represents only a part of that which has been absorbed, since other tissues, and particularly the excreta, are concerned. Nevertheless it has been shown elsewhere (2) that the amount of mercury retained, for example, by the kidney in 24 hours, may represent from 30 to 40 per cent of the total amount in the remainder of the carcass. When one considers that the kidney and the liver together make up approximately $\frac{1}{20}$ of the body weight, the concentrating effect of these tissues on mercury is at once apparent.

METHOD. Young female rats were used. They were lightly anesthetized, a small pledget of cotton inserted into the vagina and a solution of phenylmercuric acetate injected into the cotton. The use of the cotton pledget was optional, being necessary only in those cases where fluid instillations greater than 0.05 cc. were applied. The animals were placed in specially designed (3) leather holders fastened to the bottom of the cage, and confined thus for 24 hours. At the end of this time they were sacrificed and the liver and kidney tissues analyzed for mercury (4). In all but one or two instances the cotton pledgets were retained for the full 24 hours. For most of the work an aqueous 0.05 per cent solution of phenylmercuric acetate was prepared. This is the same concentration used in a commercial preparation in which the chemical is dispersed in a jelly. An attempt was made to use doses in the rat comparable to those in the human. Assuming an application of 4 gm. of jelly per average 50-kgm. individual, this amounts to 24 microgm. of mercury per kgm. In order to achieve the same level in the rat, it would require only 0.02 cc. of a 0.05 per cent solution. Such amounts proved to be too small for practical operations and therefore a somewhat larger amount, 9 microgm. per average 250-gm. rat, was set as the lowest level. This was 50 per cent larger than the average human dose, but still well below the amount which could produce vaginal irritation. Because of mechanical losses the amount of fluid injected into the vagina of the rat had to be limited to 0.1 cc. In some of the later experi-

ments, where larger amounts of mercury were used, it was necessary to increase the concentration to 0 2 per cent These higher concentrations caused moderate to severe irritation to the mucosa.

RESULTS. In table I are shown the results of a comparison between a commercial preparation of phenylmercuric acetate in a jelly, and an aqueous solution—both at 0 05 per cent concentration. With the mercury content of the liver and kidney as a relative measure of absorption, it is at once apparent that significant amounts of mercury have been absorbed by the vaginal mucosa and this observation is emphasized by the fact that control animals treated in a like manner, except for the substitution of distilled water, showed only occasional

TABLE I

Amount of mercury found in the livers and kidneys of individual rats immediately following a 24-hour vaginal exposure to a 0 05 per cent solution of phenylmercuric acetate
(0 microgm mercury per rat)

IN AQUEOUS SOLUTION				IN WATER SOLUBLE JELLY				CONTROL		
Liver	Kidney	Total	Amount injected	Liver	Kidney	Total	Amount injected	Liver	Kidney	Total
microgm mercury			*per cent*	*microgm mercury*			*per cent*	*microgm mercury*		
1 1	0 65	1 7	19	1 7	1 0	2 7	31	0	0	0
0 73	0 37	1 1	12	1 2	0 87	2 1	22	0	Trace*	Trace
1 7	0 77	2 4	27	1 3	0 75	2 0	22	0	0	0
2 0	3 0	5 0	54	1 3	1 5	2 8	31	0	Trace	Trace
1 6	2 1	3 7	41	1 6	1 2	2 8	31	0	0	0
1 1	0 78	1 9	20	0 20	1 0	1 2	14	0	Trace	Trace
1 5	0 70	2 2	24	1 7	1 7	3 4	37			
				1 7	0 75	2 5	27			
Average			28							
				Average			27			

* A quantity of mercury of the order of 0 1 microgm or less

traces of mercury in the tissues under examination. It is clear also that the jelly preparation neither enhanced nor interfered with the absorption of mercury. Because of the considerable variability in the individual storage responses of the animals included in these assay groups, differences between averages would have to be of the order of 25 per cent before they could be considered significant ($p = 0\ 05$).

The blood supply to the vaginal mucosa is closely controlled by the oestrus cycle of the animal. It may be assumed therefore that during oestrus, when the mucosa is engorged with blood, a more favorable condition for absorption might be obtained Accordingly, six animals were exposed during oestrus and six during anoestrus The results are shown in table II. It can be seen that the per cent absorption of mercury, as measured by the content of the metal in the liver and kidney, remains unaffected by the condition of the vaginal mucosa.

Since the smallest quantity of mercury administered on a per kgm. basis was

50 per cent greater in the rat than in the human, it became of interest to define the relationship between the amount of mercury instilled into the vagina and the amount recovered in the liver and kidney tissue. This is shown in table III. It can be seen that whereas the total amount of stored mercury tended to increase in an orderly fashion with increase in amount applied, the per cent increase in no way followed the same pattern. This may be accounted for by the observation that larger amounts of mercury produce vaginal irritation. Such a reaction may reduce the absorptive capacity of this tissue.

TABLE II

The effect of oestrus and anoestrus on the absorption of mercury from the vaginal mucosae of [illegible] individual rats. (9 microgm. mercury per rat in water-soluble gelatin)

OESTRUS				ANOESTRUS			
Liver	Kidney	Total	Amount injected	Liver	Kidney	Total	Amount injected
microgm. mercury			*per cent*	*microgm. mercury*			*per cent*
1.5	1.5	3.0	33	1.2	1.5	2.7	30
1.9	1.2	3.1	34	1.7	1.3	3.0	33
2.6	1.3	3.9	42	6.9	2.0	2.9	32
1.6	0.75	2.3	26	1.7	1.2	2.9	32
2.2	1.4	3.6	46	2.7	1.4	4.1	45
2.3	1.4	3.7	40	2.9	1.3	4.2	46
Average......................			37	Average......................			36

TABLE III

*Relation between the amount of mercury in contact with the vaginal mucosa and the amount which is recovered in the liver and kidney in 24 hours**

Micrograms mercury injected.........	9	18	36
Micrograms mercury found in liver and kidney tissue..................	2.8	4.1	8.8
Per cent recovered..................	31	23	24

* Average of 12 rats at each level of exposure.

In view of the repeated use of phenylmercuric acetate as a contraceptive, it becomes of interest to determine at what rate stored mercury is discharged from the liver and kidney after a single application. At the end of the 24-hour period at which the rats were usually sacrificed, the vaginal tract was carefully washed to free it as far as possible from residual amounts of mercury. The animals were then released from their holders and allowed to live 24, 48 and 168 hours after the end of the exposure. At the end of this time they were sacrificed and liver and kidneys analyzed for mercury.

The results can be seen in table IV. It is apparent that once the mercury has entered the organism, its removal is at best slow. This is particularly true for the kidney, where, depending on the dose, from 50 to 80 per cent of the stored mercury is still present after a week.

The rate at which mercury accumulates in liver and kidney tissue during 24 hours, expressed as per cent of the total amount applied, is shown in figure 1. It can be seen that the most rapid absorption of mercury occurs at the 15-microgm. level. Here absorption is 75 per cent complete within the first 8 hours.

TABLE IV

Decrease with time of stored mercury in liver and kidney. Vaginal exposure 24 hours

18 MICROGRAMS MERCURY INJECTED					36 MICROGRAMS MERCURY INJECTED				
Hrs after end of 24 hr. exposure	Liver*	Kidney*	Total	Per cent	Hrs after end of 24-hr. exposure	Liver*	Kidney*	Total	Per cent
	microgm. mercury					*microgm. mercury*			
0	1.9	2.4	4.3	24	0	3.7	5.6	9.3	26
24	1.7	2.6	4.3	24	24	1.8	4.0	5.8	16
48	0.83	2.7	3.5	19	48	1.6	5.7	7.3	20
168	0	2.1	2.1	12	168	0.98	3.6	4.6	13

* Average of 4 rats on each dose and at each period.

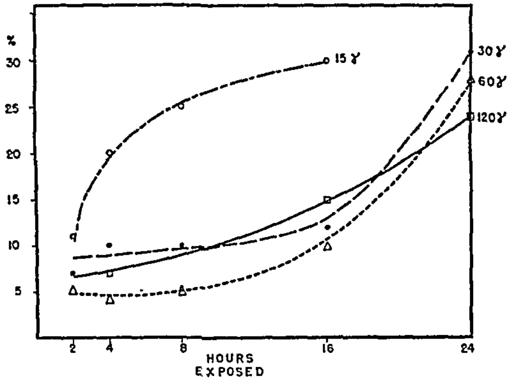


FIG. 1. PER CENT OF THE TOTAL AMOUNT OF MERCURY AT VAGINAL INSTILLATION LEVELS OF 15, 30, 60 AND 120 MICROGRAMS PER RAT WHICH IS STORED IN THE LIVER AND KIDNEYS IN 2, 4, 8, 16 AND 24 HOURS

In contrast, the lag in absorption during the first 16 hours is clearly marked at the 30, 60 and 120-microgm. levels. At these higher levels, it is not inconceivable that some interference with absorption may stem from irritative effects on the vaginal mucosa.

Gross irritation of the vaginal mucosa has also been observed in rabbits when concentrations higher than 0.05 per cent phenylmercuric acetate were injected.

However, preliminary experiments on rabbits have completely substantiated all of the results on rats and indicate that the remarkable absorption of phenylmercuric acetate from the vagina is not limited to the rat.

DISCUSSION. The results here presented serve to point out the error of assuming that the urinary excretion of mercury is a true measure of the absorption of this heavy metal. When small quantities of mercury are absorbed, they are stored and held with great tenacity, particularly by the kidney. This is not only borne out by the experiments reported in this paper, where 60 to 80 per cent of the mercury originally stored in the kidney is still present in this organ one week after a single vaginal exposure, but also by some preliminary studies on oral administration of phenylmercuric acetate (5). The results of this last investigation indicate a 25 per cent storage residue of mercury in the kidney one month after institution of a mercury-free diet. Although the quantity of mercury applied to the vaginal tract as a spermaticide is extremely small (24 microgm. per kgm.), this in itself is of little importance. Rather, consideration should be given to the fact that from every exposure, a measurable amount of mercury is stored in the kidney, and in the case of the human, repetitive exposure over a period of years is possible.

SUMMARY

A method has been presented which shows that about 25 per cent of a dose of phenylmercuric acetate when instilled into the vagina of the rat can be absorbed and stored by the liver and kidney.

REFERENCES

1. EASTMAN, N. J., AND SCOTT, A. B.: Human Fertility, 9: 33, 1944.
2. LAUG, E. P., VOS, E. A., UMBERGER, E. J., AND KUNZE, F. M.: THIS JOURNAL, 89: 42, 1947.
3. UMBERGER, E. J.: J. Lab. and Clin. Med., 31: 369, 1946.
4. LAUG, E. P., AND NELSON, K. W.: J. Assoc. Official Agr. Chemists, 25: 399, 1942.
5. LAUG, E. P., KUNZE, F. M., AND FITZHUGH, O. G.: Fed. Proc., 6: 349, 1947.

A QUANTITATIVE METHOD FOR THE DETERMINATION OF ANTI-HISTAMINIC COMPOUNDS CONTAINING THE PYRIDINE RADICAL[1]

ELY PERLMAN

Division of Bacteriology and the Medical Services, The Mount Sinai Hospital, New York, N. Y.

Received for publication December 15, 1948

Since the initial studies of Bouvet and Staub (1) hundreds of antihistaminic compounds have been synthesized (2, 3). Although a dozen or more of these substances have become widely used for the treatment of allergic diseases, very little is known concerning their metabolism. Gelvin, McGavack and Drekter (4) utilized a modification of the Brodie test (5) for organic bases and reported on the change of concentration of these bases in the blood, urine and spinal fluid of patients receiving diphenhydramine·HCl (Benadryl) or tripelennamine·HCl (Pyribenzamine). So far as this author has been able to ascertain no experiments have been published demonstrating antihistaminic activity in the body fluids of patients or experimental animals receiving antihistaminic drugs, even by means of the very sensitive pharmacological methods.

Fleming and Rieveschl have synthesized Benadryl containing radioactive C^{14} (6) and their collaborators published a brief report on the distribution of this material in the viscera of the rat and guinea pig (7).

In a search for a specific chemical method for the determination of at least some of the antihistaminic compounds, the well known cyanogen bromide test for pyridine was considered (8), especially since many antihistaminic drugs contain this radical. While the addition of cyanogen bromide to Pyribenzamine does not cause the development of the anticipated color, it was discovered that the resulting compound exhibited a brilliant blue fluorescence in filtered ultraviolet light. During the course of these studies the same test was mentioned in a review article by Huttrer (9). Mizzoni who described this method was unable to detect thereby the presence of Pyribenzamine in the blood of dogs several minutes after the intravenous administration of relatively large amounts of the drug (10).

In the experiments to be described, the cyanogen bromide test was applied to all the available antihistaminic compounds containing the pyridine radical in order to select those most suitable for further study. A survey of related compounds was made to ascertain the specificity of the method and optimal conditions for the test were determined. The application of this technic to the determination of the urinary excretion of the drugs will be described. Evidence for the chemical nature of the urinary excretion product of Pyribenzamine will be presented and discussed.

[1] This investigation was aided by grants from the Elsa and William Menke Fund and from the Lasdon Foundation, Inc.

Experimental. I. *Survey of antihistaminic compounds containing pyridine.* Solutions containing 1000, 100 and 10 microgm./cc. of each of the following substances were prepared in distilled water: doxylamine (Decapryn succinate[2]), pyranisamine (Neo-Antergan maleate[3]), tripelennamine (Pyribenzamine HCl[4]), chlorothen (Tagathen HCl[5]), methapyrilene (Thenylene HCl[6]), prophenpyridamine (Trimeton maleate[7]), and Win 2848-2 HCl.[8] Included for comparison were diphenhydramine (Benadryl HCl[9]), Diatrin HCl,[10] thonzylamine (Neohetramine HCl[11]), Phenargan HCl,[12] phenindamine (Thephorin tartrate[13]), nicotinic acid, pyridine and 2-aminopyridine. The purity of these compounds was assumed. Four cc. samples of the 3 concentrations of each were mixed with 2 cc. of a saturated aqueous solution of cyanogen bromide prepared just prior to use from the crystalline compound (Eastman Kodak Co.). After ½ hour the solutions were observed for the development of fluorescence under ultraviolet light. The intensity of the fluorescence produced by each compound is recorded in table I. It can be seen from this table that several of the compounds developed a color instead of a fluorescence. The same experiment was repeated with all of the substances but 1 cc. of a ¼ per cent solution of p-aminoacetophenone (11) was added prior to the addition of the CNBr. This coupling compound served to intensify the color developed but did so only with those substances which produced some color with the CNBr alone. These results are included in table I.

All of the materials which developed a fluorescence emitted a blue color similar to that of thiochrome. Spectroscopic examination however revealed no sharp maximum. As in the case of thiochrome, the fluorescence of the reaction product of Pyribenzamine with CNBr is reversibly quenched by reducing agents. Thus, the addition of a 10 per cent solution of sodium hydrosulfite in 5 per cent sodium bicarbonate destroys the fluorescence which is then readily regenerated by the addition of hydrogen peroxide or other oxidizing agents.

From the results shown in table I it is apparent that only those compounds with a nitrogen atom in the position ortho to the pyridine nitrogen developed a fluorescence with CNBr. Those drugs which contain pyridine but do not have this additional nitrogen atom in the molecule produce a color with the CNBr instead of a fluorescence. This was true, as expected, for both nicotinic acid and pyridine itself. The nitrogen in the ortho position is necessary for fluorescence, but not sufficient since 2-aminopyridine does not develop a fluorescence.

II. *Quantitative fluorescence measurements.* In order to obtain more accurate comparisons of the potentially fluorescent compounds, the method to be described was used.

Five cc. samples of $M/15$ phosphate buffer pH 6.6 containing various concentrations of the antihistaminic compounds (as the acid salt) were mixed with 2 cc. of a freshly prepared

---

[2] Supplied by Wm. S. Merrell Co., Cincinnati, Ohio.
[3] Supplied by Merck & Co., Inc., Rahway, N. J.
[4] Supplied by Ciba Pharmaceutical Products, Inc., Summit, N. J.
[5] Supplied by Lederle Laboratories, Pearl River, N. Y.
[6] Supplied by Abbott Laboratories, North Chicago, Ill.
[7] Supplied by Schering Corp., Bloomfield, N. J.
[8] Supplied by Winthrop-Stearns, Inc., N. Y., N. Y.
[9] Supplied by Parke Davis & Co., Detroit, Mich.
[10] Supplied by William R. Warner & Co., N. Y., N. Y.
[11] Supplied by Wyeth Inc., Philadelphia, Pa.
[12] Supplied by Merck & Co., Inc., Rahway, N. J.
[13] Supplied by Hoffmann-LaRoche Inc., Nutley, N. J.

TABLE 1

A comparison of the property of various antihistaminic compounds to develop either a fluorescence or a color with cyanogen bromide

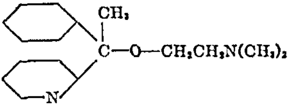
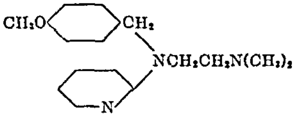
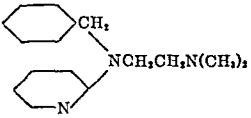
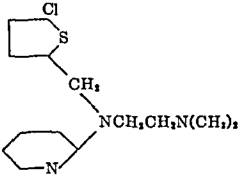
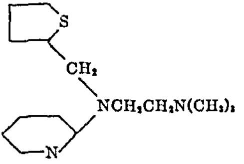
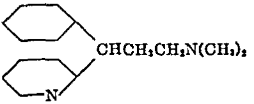
No in text	Compound	Formula of free base	Intensity of fluorescence	Color developed with: CNBr alone	Color developed with: Coupling agent
1	Decapryn succinate		0	Yellow	Pink, yellow, 3+, fades
2	Neo-antergan maleate		1+	0	Very faint yellow
3	Pyribenzamine hydrochloride		4+	0	0
4	Tagathen hydrochloride		2+	0	0
5	Thenylene hydrochloride		3½+	0	0
6	Trimeton maleate		0	Yellow	Yellow 2+, fades rapidly

TABLE 1—*Continued*

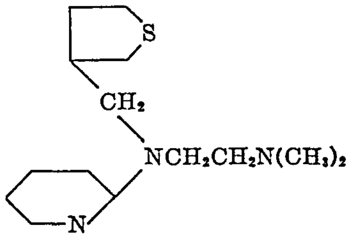
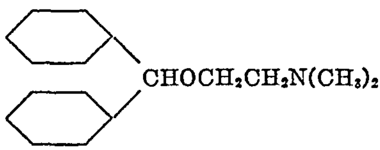
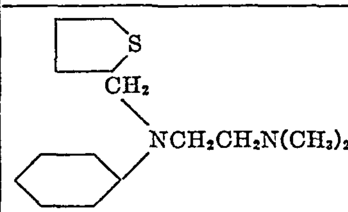
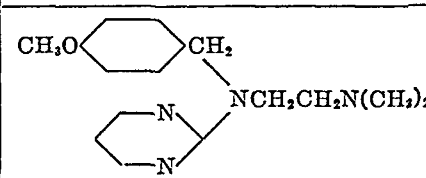
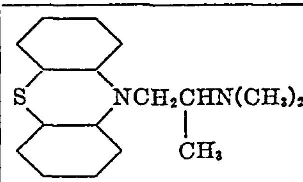
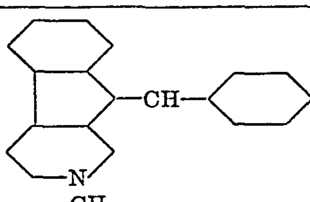
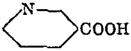
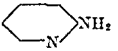
NO. IN TEXT	COMPOUND	FORMULA OF FREE BASE	INTENSITY OF FLUORESCENCE	COLOR DEVELOPED WITH	
				CNBr alone	Coupling agent
7	Win 2848-2 hydrochloride		4+	0	0
8	Benadryl hydrochloride		0	0	0
9	Diatrin hydrochloride		0	0	0
10	Neohetramine hydrochloride		0	0	0
11	Phenargan hydrochloride (RP 3277)		0	Pink	Blue
12	Thephorin tartrate		0	0	Turbid

TABLE 1—*Continued*

NO. IN TEXT	COMPOUND	FORMULA OF FREE BASE	INTENSITY OF FLUORESCENCE	COLOR DEVELOPED WITH	
				CNBr alone	Coupling agent
13	Nicotinic acid		0	Yellow	Yellow 4+, stable
14	Pyridine		0	Orange	Yellow 4+, stable
15	2-aminopyridine		0	Yellow	Yellow 2+, stable

saturated solution of CNBr. The mixtures were allowed to stand for ½ hour and the intensity of the fluorescence developed was measured in a Pfaltz and Bauer Model A Fluorophotometer using the filters provided for thiochrome determinations. (Ultraviolet filter with a maximum transmission at 370 mμ for the light source and a combination of No. 038 yellow and No. 428 blue with a maximum at 460 mμ for the photocell). The sensitivity of the instrument was adjusted so that a permanent glass thiochrome standard would give a reading of 10 on the galvanometer.

Figure 1 graphically summarizes the results of these observations. In this figure the galvanometer readings are plotted against the concentration of drug. It can be seen that all of the compounds with a single exception of Neo-Antergan *show a linear relationship between the intensity of fluorescence (galvanometer* readings) and the concentration of the material used. The compounds are listed in order of descending intensity of developed fluorescence; Win 2848-2 HCl = Pyribenzamine HCl > Thenylene HCl > Tagathen HCl > Neo-Antergan maleate. If the graph of fig. 1 is redrawn using moles as the abscissa instead of weight, the above order is not altered.

III. *Method and application of the test to urine.* If the urine from a patient receiving Pyribenzamine is treated with alkali and shaken with an organic solvent (as in the Gelvin, McGavack and Drekter (4) modification of the Brodie test) the material obtained in the organic solvent does not fluoresce after the addition of CNBr. It was found that heating the urine after the addition of alkali liberated a substance (or substances) which was then soluble in benzene or other organic solvents. This substance developed a fluorescence with CNBr. Based upon this observation, the following method for the quantitative determination of these antihistaminic compounds was developed.

In general and unless otherwise specified, the test was performed in the following manner: 5 cc. of the subject's urine was placed in a glass stoppered 15 cc. centrifuge tube and made alkaline with 0.5 cc. of a 10 per cent solution of NaOH. The tube containing the alkaline urine was placed in a boiling water bath for 20 minutes, after which it was quickly cooled. Five cc. of benzene was then added and the tube stoppered and shaken vigorously 25 times.

Four cc. of the upper benzene layer was then transferred to another tube of the same type. This benzene solution was then shaken with 1 cc. of $N/2$ HCl. The lower aqueous layer was removed as completely as possible with a glass capillary and was transferred to a 15 cc. graduated test tube. The benzene solution was extracted a second time with 1 cc. of $N/20$ HCl and this was transferred in the same manner to the graduated test tube. The contents of the latter were then neutralized with $N/1$ NaOH and the volume made up to

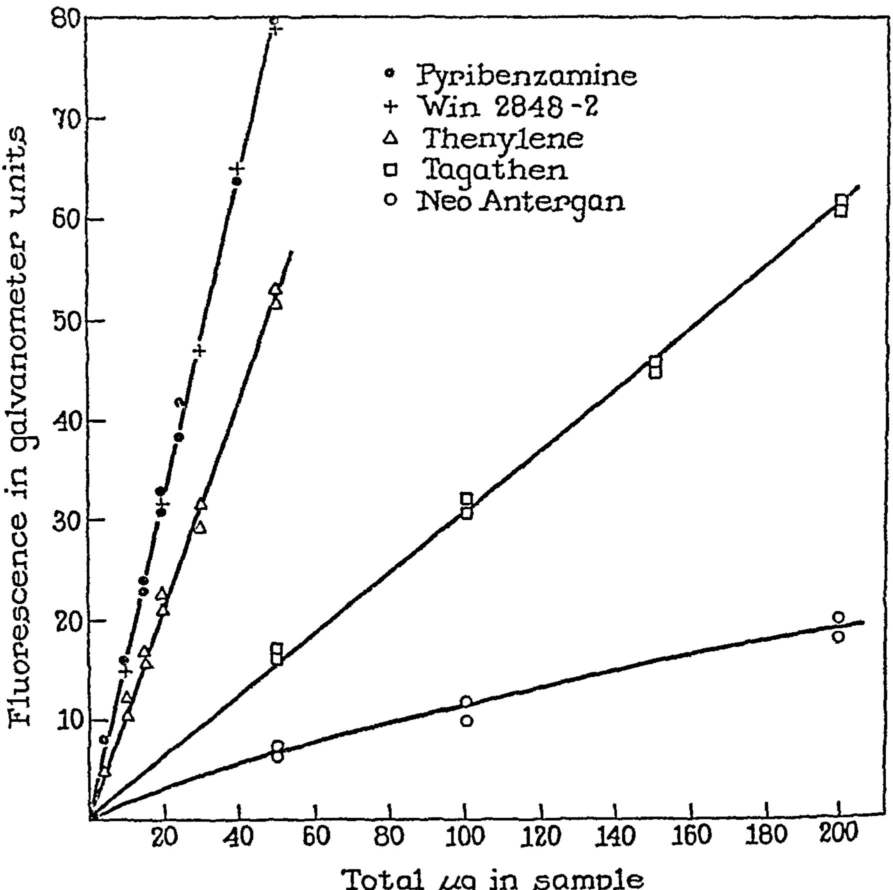


FIG. 1. THE INTENSITY OF FLUORESCENCE DEVELOPED BY VARIOUS CONCENTRATIONS OF SEVERAL ANTIHISTAMINIC COMPOUNDS

5 cc. by the addition of sufficient $M/15$ phosphate buffer of pH 6.6. To this was added 2 cc. of a freshly prepared saturated solution of CNBr. After ½ hour the contents were transferred to the specially selected fluorophotometer tubes and the fluorescence measured as described above in Section II.

The fluorescence developed is measured in ½ hour since it was found that after the addition of the CNBr the fluorescence increases rapidly reaching a maximum within this period of time and remains constant for at least 18 hours thereafter. Figure 2 illustrates the rate of development of fluorescence of several different concentrations of Pyribenzamine added to normal urine.

The amount and concentration of each of the reagents prescribed for the test were found

to provide a sufficient excess to insure maximum development of fluorescence. The benzene extraction and the subsequent acid washes were likewise found to yield maximum values. This holds true only for the range of concentrations of Pyribenzamine herein studied.

Solutions of Pyribenzamine were prepared in $N/20$ HCl, $N/20$ NaOH and in $M/15$ phosphate buffers through a pH range of 6.0 to 8.0. The fluorescence developed after the addition of CNBr was the same in all instances with the single exception of the solution in $N/20$ HCl which developed no fluorescence at all. The addition of strong acid or alkali to the solutions after the fluorescence had already been developed caused no change to occur. These results were found to be true for Thenylene HCl and for Neo-Antergan maleate as well.

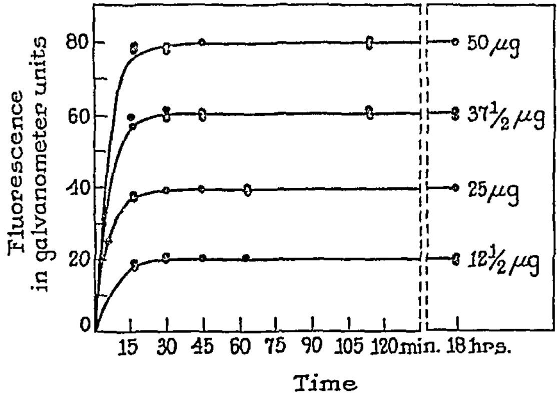


Fig. 2. The Development of Fluorescence with Time

The heating of the urine of patients receiving Pyribenzamine was found to be a critical step in the test. The following experiments were done to determine the optimal conditions.

Samples of urine from several patients receiving Pyribenzamine orally were tested as described above with the exception that, after the addition of the alkali, 5 cc. aliquots of each were heated in the boiling water bath for various periods of time. They were then extracted with benzene and the test was completed in the usual manner. The results of one such experiment are recorded in fig. 3 and are typical The yield of fluorescent material starts at zero and increases rapidly during the first 10 minutes of heating. It reaches a maximum in 15 minutes and remains approximately the same for the next 10 minutes after which destruction begins and the fluorescence decreases slowly but significantly.

Pyribenzamine HCl was added to a normal urine to give a final concentration the same as that estimated to be present in the patient's urine just described. This urine was made alkaline and was divided into 5 cc. aliquots which were heated in the boiling water bath for various periods of time before the completion of the test. The results are shown in fig. 3 along with those of the previous experiment. The fluorescence in this instance begins, of course, at a maximum and remains unchanged for the first 15 minutes, after which significant destruction of the material begins.

Four cc. of the upper benzene layer was then transferred to another tube of the same type. This benzene solution was then shaken with 1 cc. of $N/2$ HCl. The lower aqueous layer was removed as completely as possible with a glass capillary and was transferred to a 15 cc. graduated test tube. The benzene solution was extracted a second time with 1 cc. of $N/20$ HCl and this was transferred in the same manner to the graduated test tube. The contents of the latter were then neutralized with $N/1$ NaOH and the volume made up to

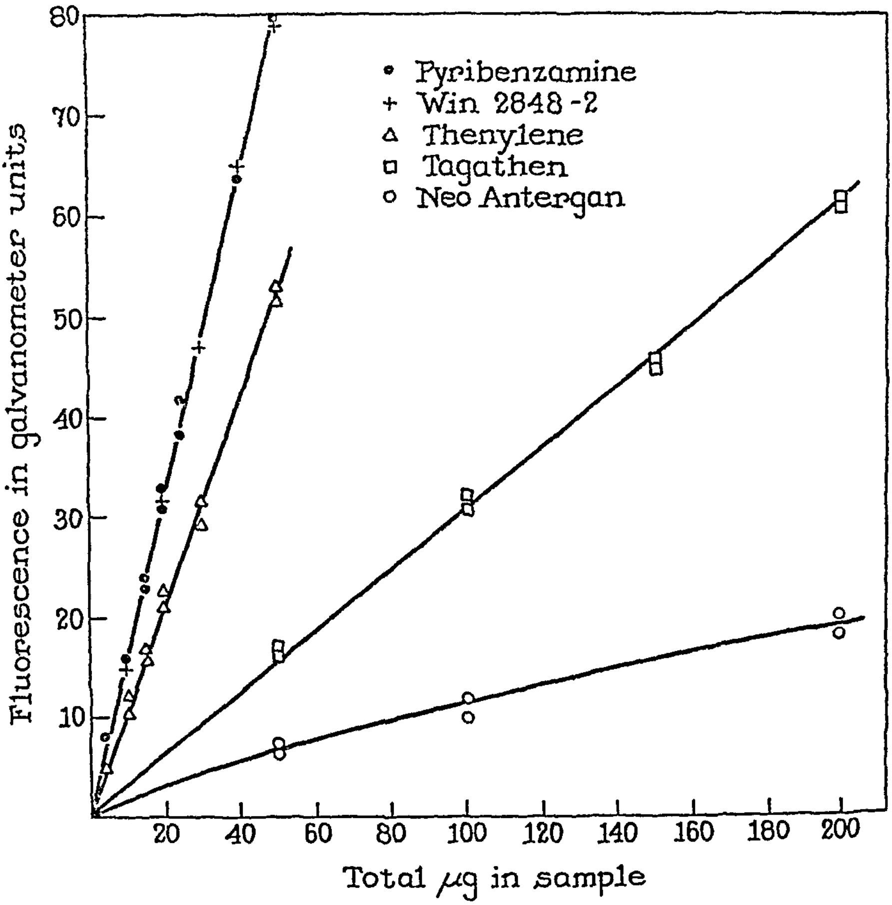


FIG. 1. THE INTENSITY OF FLUORESCENCE DEVELOPED BY VARIOUS CONCENTRATIONS OF SEVERAL ANTIHISTAMINIC COMPOUNDS

5 cc. by the addition of sufficient $M/15$ phosphate buffer of pH 6.6. To this was added 2 cc. of a freshly prepared saturated solution of CNBr. After ½ hour the contents were transferred to the specially selected fluorophotometer tubes and the fluorescence measured as described above in Section II.

The fluorescence developed is measured in ½ hour since it was found that after the addition of the CNBr the fluorescence increases rapidly reaching a maximum within this period of time and remains constant for at least 18 hours thereafter. Figure 2 illustrates the rate of development of fluorescence of several different concentrations of Pyribenzamine added to normal urine.

The amount and concentration of each of the reagents prescribed for the test were found

Five of the antihistaminic compounds which did not develop a fluorescence with CNBr were given to as many volunteers to determine whether their urinary excretion products would develop this property. Thus, Decapryn, Trimeton, Diatrin, Neohetramine and Thephorin were tried. The urine of each volunteer was tested by the CNBr method and all proved to be negative. However, those compounds which develop a color upon the addition of CNBr (Decapryn and Trimeton) also showed the development of the same color with their urinary excretion products.

V. *A comparison of the CNBr test with the Brodie test for organic bases.* When the Gelvin, McGavack and Drekter modification of the Brodie test was applied to the determination of the concentration of organic bases in the urine of subjects receiving Pyribenzamine, the values obtained were found to be considerably lower than those by the CNBr test as described above. The obvious difference between the two methods is the heating of the urine in the latter. In order to clarify this discrepancy, the following experiment was performed on aliquots of a single sample of urine from a patient receiving Pyribenzamine.

a) Four 5 cc. portions of this urine were each treated with 0.5 cc. of 10 per cent NaOH. Two were heated for 20 minutes in a boiling water bath, the other 2 were not. Each was then shaken with 5 cc. of benzene to which 0.1 cc. of isoamyl alcohol was added. Four cc. of this benzene layer was transferred to another tube and shaken with 0.4 cc. of $N/2$ HCl and 8 times with 0.4 cc. of $N/20$ HCl. These accumulated washings were made up to 10 cc. with distilled water. The color was measured in a Coleman Universal Spectrophotometer at 510 mμ. The transmission values were converted into concentrations by means of reference to a standard curve. The standard curve was prepared by adding known amounts of Pyribenzamine HCl to 5 cc. aliquots of a normal urine and submitting these to the test just described.

b) The same urine sample used in a) was divided into another four 5 cc. portions and each was treated with 0.5 cc. of 10 per cent NaOH. Two were not heated, the other 2 were placed in a boiling water bath for 20 minutes. Each was submitted to the standard CNBr test. The galvanometer readings obtained were converted into concentrations by means of reference to a standard curve.

The results of these comparisons are shown in table 2. These results are identical with those obtained in a similar set of experiments in which ethylene dichloride was substituted for the benzene. It is seen that with the Methyl Orange test, which gives low values (3–4 microgm./cc.), the addition of heating increases the values approximately ten-fold. The values obtained after heating are then about the same or but slightly higher than those obtained with the CNBr test.

Experiments were done to determine whether the organic base that is extracted without heating (as in the Brodie test) contains some conjugated compound of Pyribenzamine. Although the details are not recorded, it was found that the compound in the urine which fluoresces is not extractable with benzene until it is heated with alkali.

It was considered of interest to determine whether this compound occurs in the urine in combination with a protein or other large molecule.

Therefore, a 10 cc. sample of urine from a patient receiving Pyribenzamine was placed in a dialyzing sac (cellophane-Visking Corp.) and dialyzed overnight at 4°C. against 20 cc.

of $M/15$ phosphate buffer of pH 6.6. Aliquots of the contents of the sac and of the dialysate were analyzed by the CNBr method. However, one portion of each was heated with alkali. The other remained unheated. The samples which were not heated failed to develop any fluorescence, indicating that no spontaneous dissociation had occurred. The original solution in the dialyzing sac contained 40 microgm./cc. before dialysis, and after dialysis, was found to contain 14 microgm./cc. The buffer solution contained 13 microgm./cc.

These results indicate an even distribution of the Pyribenzamine metabolite and a ready dialysis of the material without dissociation.

VI. *Antihistaminic activity of the compound obtained from urine.* To the author's knowledge, it has heretofore been impossible to demonstrate by means of bioassay, the presence of any substance in the blood or urine of patients taking antihistaminic medication which has any specific antihistaminic activity. It

TABLE 2

A comparison of the results obtained with a subject's urine using both the Brodie test and the cyanogen bromide test on heated and unheated samples

URINE SAMPLE	ALKALI PLUS	BRODIE TEST		CNBr TEST	
		T	Concentration	Reading	Concentration
		per cent	*microgm./5 cc.*		*microgm./5 cc.*
A1	—	90	3	—	—
A2		87	4		
B1	Heat	39	48	—	—
B2		42	44		
C1	—	—	—	1	0
C2				0	0
D1	Heat	—	—	46	46
D2				44	44

was therefore of interest to determine whether an active material could be obtained from such a urine by means of the extraction procedure as used in the test.

Accordingly, urine samples obtained from patients receiving Pyribenzamine were pooled to make a total of 500 cc. Fifty cc. of 10 per cent NaOH were added and the urine boiled for 20 minutes. The urine was extracted with an equal volume of benzene. The benzene layer was removed and was washed twice with 250 cc. of water containing 25 cc. of 10 per cent NaOH. The benzene was then concentrated by evaporation in the cold to a volume of 25 cc. This material was then extracted with 2 cc. of $N/2$ HCl and again with 2 cc. of $N/20$ HCl. The extracts were pooled, neutralized and made up to 7 cc. by the addition of $M/15$ phosphate buffer of pH 6.6. This is referred to as *solution A* and was found to contain 2.7 mgm./cc. of Pyribenzamine hydrochloride by the standard CNBr test. A control solution (*solution B*) was prepared by treating 500 cc. of normal urine in the same fashion. A positive control was made by adding Pyribenzamine hydrochloride to an aliquot of solution B to give a final concentration of 2.7 mgm./cc. (as found in solution A). This is referred to as *solution C*. A solution D containing 2.7 mgm./cc. of Pyribenzamine hydrochloride in physiological saline was prepared as a reference standard.

Each of these solutions was then tested for its antihistaminic activity through the kindness of Dr. John V. Scudi of the Pyridium Corporation. The test used was that of Castillo and de Beer (12) in which the ability of the solution to relax a histamine-induced spasm in a guinea pig's tracheal chain is measured. Each solution was diluted appropriately (1:10,800) to give a final concentration of about 0.25 microgm./cc. just before testing.

The results are summarized in table 3 where it is apparent that the activities of solutions A, C and D are essentially the same within the limits of the experimental error of this method. The extractives from the urine of patients receiving Pyribenzamine (solution A) not only exhibited antihistaminic activity, but did so to the extent estimated from the CNBr test.

In view of the antihistaminic activity shown by this urinary excretion product of Pyribenzamine, it can be inferred that the dimethyl ethylenediamine portion of the Pyribenzamine molecule is intact. From the fact that the material develops a fluorescence with CNBr, it would likewise appear that the pyridine portion of the molecule is not only intact, but remains attached to the dimethyl ethylenediamine radical.

TABLE 3

The antihistaminic activity of the material obtained from the urine of a subject receiving Pyribenzamine orally

PREPARATION	RELAXATION OF HISTAMINE SPASM
	per cent
A unknown	80
B* negative urine control	0
C Positive urine control	67
D standard	60

* Solution B caused a slow relaxation. This non-specific spasmolytic action was readily differentiated from the rapid relaxation induced by the other solutions. This effect was small and was taken into account by reference to the positive urine control C.

VII. *Identification of the urinary excretion product of Pyribenzamine.* In a further attempt to prove the identity of the material obtained from the urine of patients receiving Pyribenzamine with Pyribenzamine itself, two additional experiments were performed. Material for these studies was obtained by preparing another solution from 800 cc. of urine in the same manner as described for Solution A in section VI above.

a) *Isolation as the picrate.* Seven cc. of the final solution estimated to contain a total of approximately 7 mgm. of Pyribenzamine was treated with a slight excess of a saturated aqueous solution of picric acid. The resulting precipitate was recrystallized 4 times from acetone. The hexagonal crystals melted sharply at 178°C. (uncorrected) without decomposition. Crystals of Pyribenzamine picrate were obtained in a similar manner from a solution of Pyribenzamine hydrochloride. After 4 recrystallizations, its melting point was found to be the same. Mixed crystals were prepared and revealed no depression in the melting point.

b) *Ultraviolet absorption spectrum.* The remainder of this solution was submitted to ultraviolet spectrophotometric analysis at two different values of pH. The solution was

diluted 1:40 with $M/15$ acetate buffer of pH 3.6 and the same dilution was made in $M/15$ phosphate buffer of pH 8.0. Each was estimated to contain 19 microgm./cc. by the CNBr method. Two standard solutions of 25 microgm./cc. of Pyribenzamine HCl were prepared in the same buffers. Each solution was placed in an appropriate cuvette with an optical depth of 1 cm. and the optical densities were determined for wavelengths from 220 to 340 mμ. The plain buffers were used as blanks. The measurements were made with a Beckman Quartz Spectrophotometer, Model D.

The results are shown graphically in figures 4 and 5. Figure 4 shows the curves obtained with the known solution of Pyribenzamine at pH 3.6 and 8.0. The arrows indicate two maxima, one minimum and three isobestic points. Figure 5 shows the absorption curves obtained with the unknown solution.

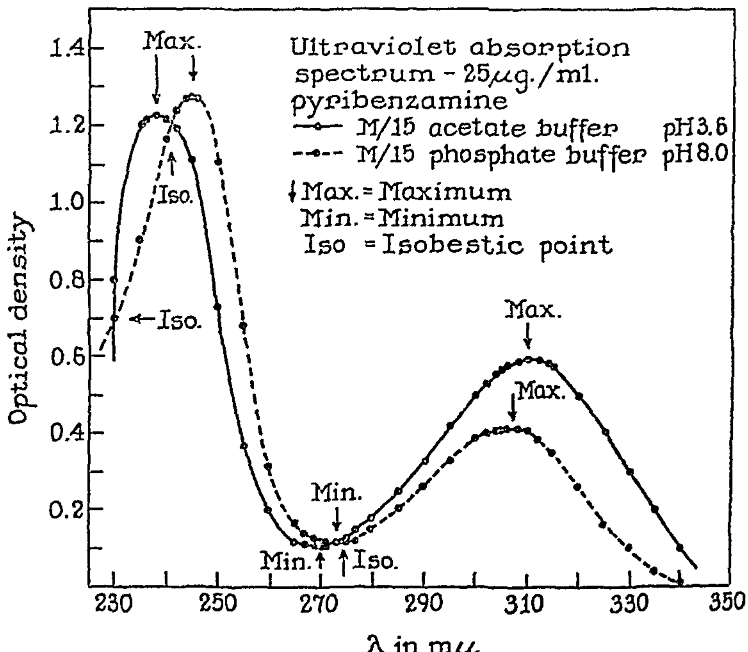


FIG. 4

Within the limits of experimental error the same maxima, minima and isobestic points are apparent. It is to be noted in addition, that the concentration of Pyribenzamine as estimated by the CNBr method (19 microgm./cc.) conforms to the concentration that can be calculated from the optical density of the unknown solution at wavelength 310 mμ (19.5 microgm./cc.). The strong absorption of Pyribenzamine at 245 mμ (pH 8.0) suggests another possible but somewhat less sensitive method for the determination of perhaps a greater variety of antihistaminic compounds.

VIII. *Preliminary studies of urinary excretion.* A group of 6 subjects volunteered for these studies. Three subjects ingested one 50 mgm. tablet of Pyribenzamine and the remaining 3 received 2 such tablets just following break-

fast. A urine sample was obtained just before taking the medication and at intervals thereafter. The total volume of each sample was noted and the concentration of Pyribenzamine was determined in each by the CNBr method. In those instances where the urine samples contained too high a concentration of

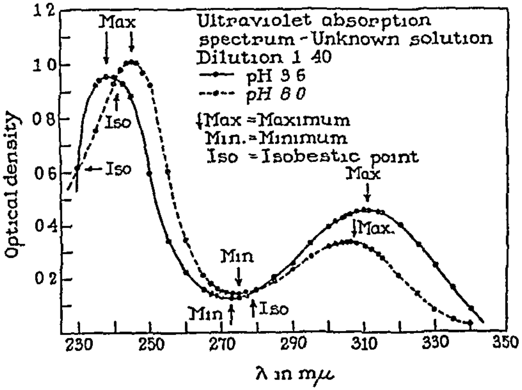


FIG 5

TABLE 4

Concentration and amount of Pyribenzamine excreted in urine following a single oral dose of 50 mgm

TIME ELAPSED	CONCENTRATION OF DRUG IN URINE	VOLUME OF URINE EXCRETED	TOTAL DRUG EXCRETED	RATE OF EXCRETION OF DRUG	CUMULATIVE TOTAL OF DRUG EXCRETED
hours	*microgm /cc*	*cc*	*microgm*	*microgm / ½ hr*	*microgm*
0	0	82	0	0	0
1	1	30	30	15	30
2	12	58	696	348	726
3	4	257	1028	514	1754
4	8	120	960	480	2714
5	13	44	572	286	3286
6	10	39	390	195	3676
8	7	48	336	84	4012
15	1 6	250	400	29	4412
22	Trace	150	—	—	—

drug the determinations were repeated using an appropriate dilution. All the determinations were done in duplicate The variations encountered never exceeded 10 per cent and were usually within 5 per cent The subjects were all ambulatory and engaged in their routine activities including eating and drinking. The results of one fairly typical experiment are recorded in table 4, whereas the results of all such determinations are summarized in figures 6 and 7.

In figure 6 the rate of urinary excretion expressed in terms of micrograms of drug excreted per ½ hour is plotted against the time elapsed following the ingestion of Pyribenzamine. In figure 7 the total amount of drug excreted is plotted against the same abscissa. It will be seen from these graphs that the

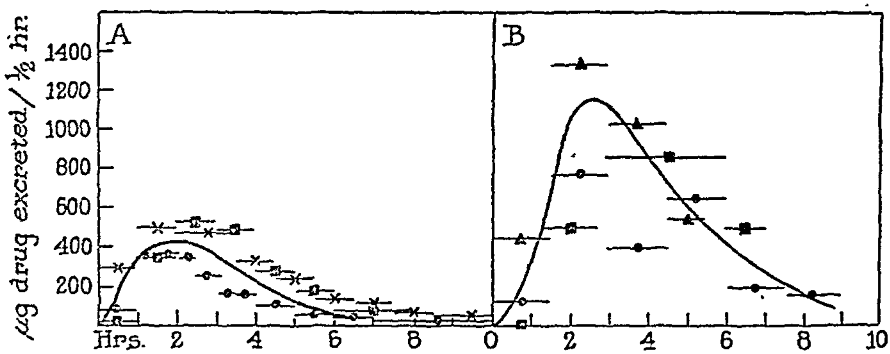


FIG. 6. THE RATE OF URINARY EXCRETION OF PYRIBENZAMINE BY SUBJECTS RECEIVING: A) 50 MGM AND B) 100 MGM. ORALLY

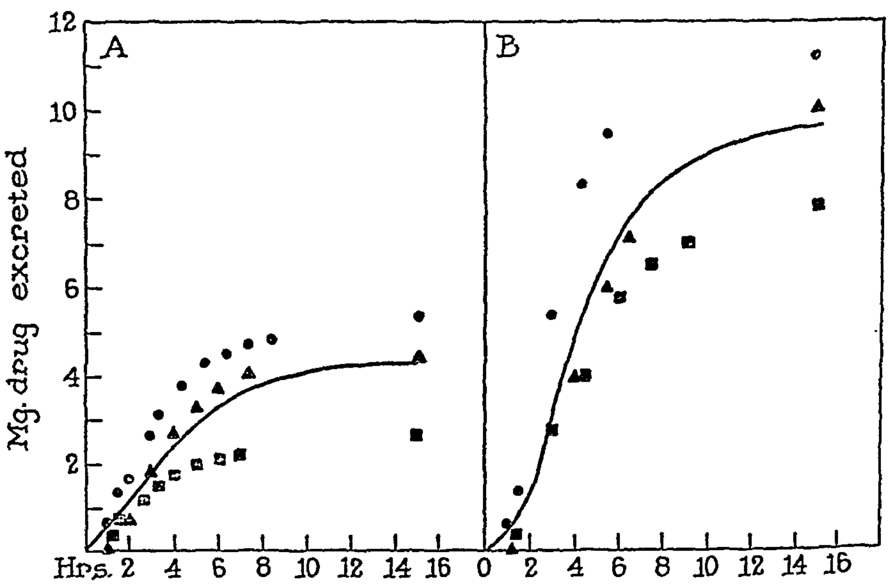


FIG. 7. THE TOTAL URINARY EXCRETION OF PYRIBENZAMINE BY SUBJECTS RECEIVING A) 50 MGM. AND B) 100 MGM. ORALLY

maximum rate of excretion occurs between 1½ and 4½ hours, while the total amount of drug excreted continues to increase significantly for some 8–10 hours, after which there is but a slight additional rise. The maximum total excretion when approximated from the asymptote to these curves is about 6–11 per cent of the amount of drug ingested.

Two subjects were studied after the ingestion of 100 mgm. of Thenylene HCl and the results in general were similar to those described above.

Twenty-five mgm. of Pyribenzamine HCl[14] were given intravenously to each of 2 subjects and the rate and total urinary excretion determined. It was found that the excretion of the drug began no sooner than that following oral administration. The curves obtained for the rate of excretion parallel those found in the cases described above, reaching a maximum between 1½ and 3½ hours. The total amount of drug excreted in the urine was approximately 8 per cent of the amount injected, as compared with 6–11 per cent excreted when the Pyribenzamine was ingested. Large volumes of all the urine samples obtained were also tested for the presence of free Pyribenzamine. This was done by testing in the usual manner with the exception that the samples were not heated in the boiling water bath. There was no significant increase in the amount of free Pyribenzamine excreted over that found after oral administration.

In both subjects it was noted that some drowsiness developed, not during the injection, but about 1–1½ hours following it.

These results suggest a few possible conclusions. It would appear that almost all of the ingested Pyribenzamine is absorbed from the gastrointestinal tract, and that regardless of the route of administration the drug is rapidly removed from the blood stream. Some 10 per cent of the medication then slowly makes its appearance in the urine where most of it is found in combination with some unknown acidic substance.

DISCUSSION. A method is described for the determination of several antihistaminic compounds which have the pyridine radical as part of their structure. The method is based upon the fact that some of these compounds develop a blue fluorescence in ultraviolet light after reacting with cyanogen bromide. It appears from an examination of such compounds that only those with a nitrogen atom in the position ortho to the pyridine nitrogen have this property. That this condition is necessary but not sufficient is evident from the fact that 2-aminopyridine does not develop a fluorescence. In addition it can be seen that not all of the antihistaminic compounds which have this structure develop the same intensity of fluorescence when equimolar concentrations are compared. These compounds differ from each other only by virtue of variations in the other aromatic radical (e.g. N-2-thenyl in Thenylene or N-p-methoxybenzyl in Neo-Antergan). While it cannot be stated that this aromatic radical is necessary for the compound to develop fluorescence, it is obvious that it does influence its intensity.[15]

[14] A special solution of Pyribenzamine HCl prepared for intravenous use was obtained through the courtesy of Dr. C. R. Scholz, Ciba Pharmaceutical Products, Inc., Summit, N. J.

[15] Since the submission of this paper for publication two additional compounds were obtained through the courtesy of Dr. C. R. Scholz. The first, 2-benzylaminopyridine developed no fluorescence with CNBr. The second, N,N-dimethyl-N′-ethyl-N′-(2-amino pyridine) does develop a fluorescence, the intensity of which is intermediate between that developed by Tagathen and Neo-Antergan. The second compound is similar to Pyribenzamine except that the benzyl radical is replaced by an ethyl group. This has obviously

The application of this test to the urine from patients receiving these medications is described. It was found that, whatever the urinary excretion product may be, it is possible by heating such a urine with alkali to extract an active substance therefrom with benzene. The material in this benzene solution develops a fluorescence upon the addition of cyanogen bromide. A variety of medications and naturally occurring compounds were tested with cyanogen bromide and failed to develop a fluorescence. This observation, in conjunction with the fact that a large number of normal urines were negative, indicates that the test is highly specific for those antihistaminic compounds described above.

A number of volunteers were given Pyribenzamine by mouth and their urinary excretion was determined by the cyanogen bromide test. It was found that the greatest rate of excretion occurs between $1\frac{1}{2}$ and $4\frac{1}{2}$ hours which coincides with the clinical observations on the peak and duration of action of this drug (e.g. 13, 14). It was further noted that the maximum amount excreted as detected by this method was about 10 per cent of the amount ingested. Two subjects received Pyribenzamine intravenously and both the rate of excretion as well as the total amount excreted were generally the same as found following oral administration.

It is concluded from these studies that most of the Pyribenzamine administered orally is absorbed from the gastrointestinal tract and that some 10 per cent is excreted in the urine as Pyribenzamine itself in firm combination with some undetermined acid or acidic radical of small molecular size. That the combination is firm is shown by the necessity of heating with alkali to accomplish liberation of the free Pyribenzamine base. That the molecular size is small is indicated by its dialysis through cellophane tubing without dissociation. Since the dissociated base has been shown to have antihistaminic activity, the dimethyl ethylenediamine portion of the molecule can be assumed to be intact. This is probably true for the methyl benzene radical as well (15). The attachment of the pyridine molecule thereto can be inferred from the fact that the material develops a fluorescence with cyanogen bromide. It is concluded that the pyridine radical has not been oxidized or otherwise irreversibly altered in view of the identity of the ultraviolet absorption spectrum of this material with that of Pyribenzamine itself. In addition, the material has been isolated as the crystalline picrate whose melting point is the same as that of a known sample of Pyribenzamine picrate.

SUMMARY AND CONCLUSIONS

1). A quantitative fluorophotometric test is described for those antihistaminic compounds containing pyridine and dimethyl ethylenediamine in the molecule. Cyanogen bromide reacts with these compounds to form a fluorescent substance.

---

resulted in a decrease in the intensity of, but not in the elimination of fluorescence. It would seem fairly certain that the condition necessary for fluorescence to develop is the presence of the three nitrogen atoms in the same positions as they occur in Pyribenzamine, i.e. dimethylethylenediamine combined with pyridine in the ortho position.

2). The test has been applied to a study of the urinary excretion of one such compound by human subjects.

3). The test is specific for these drugs since normal urines and a variety of possible interfering compounds are negative.

4). About 10 per cent of orally ingested Pyribenzamine is excreted in the urine in the form of an organic base firmly combined with some acidic substance or radical.

5). This urinary excretion product when dissociated by heat and alkali was found to have antihistaminic activity.

6). That this material is identical with Pyribenzamine is suggested by the fact that, a) its isolation as the picrate yielded crystals with a melting point the same as Pyribenzamine picrate and, b) the material has the same ultraviolet absorption spectrum as Pyribenzamine itself.

ACKNOWLEDGEMENT. The author takes pleasure in expressing his gratitude to Dr. G. Shwartzman, Dr. H. H. Sobotka and Dr. S. Jarcho for their interest and help, and to Misses R. Goidel and L. Berg for their technical assistance.

REFERENCES

1. BOUVET, D., AND STAUB, A.: Compt. rend. soc. de biol., 124: 547, 1937.
2. HUTTRER, C. P.: Enzymologia, 12: 277, 1948.
3. HALPERN, B. N.: Act. Allergol., 1: 3, 1948.
4. GELVIN, E. P., MCGAVACK, T. H., AND DRECKTER, I. J.: Bull. N. Y. Med. Coll. Flower and Fifth Ave. Hosp., 9: 51, 1946.
5. BRODIE, B. B., AND UDENFRIEND, S.: J. Biol. Chem., 158: 705, 1945; 168: 335, 1947.
6. FLEMING, R. W., AND RIEVESCHL, J., JR.: Abst. 112th Meeting A. C. S., Pg. 16k, 1947.
7. GLAZKO, A. J., DILL, W. A., AND MCGINTY, D. A.: Fed. Proc., 7: 222, 1948.
8. GORIS, A., AND LARSONNEAU, A.: Bull. Sci. Pharmacol., 23: 497, 1921. See also original Beilstein Vol. XII, pg. 555, Vol. XX, pg. 188.
9. See ref. 2, pg. 288, bottom.
10. MIZZONI, R.: Personal communication.
11. KODICEK, E.: Biochem. J., 34: 712, 1940.
12. CASTILLO, J. C., AND DE BEER, E. J.: THIS JOURNAL, 90: 104, 1947.
13. BAER, R. L., AND SULZBERGER, M. B.: J. Invest. Derm., 7: 201, 1946.
14. LOVELESS, M., AND BROWN, H.: New England J. Med., 237: 592, 1947.
15. SCHOLZ, C. R.: Abstract of paper presented at the First National Symposium for Medicinal Chemistry, Ann Arbor, Mich., June 1948. See Compound SU 111.

DETERMINATION OF GALLIUM IN BIOLOGICAL MATERIALS[1]

H. C. DUDLEY

Naval Medical Research Institute, National Naval Medical Center, Bethesda, Maryland

Received for publication December 15, 1948

Interest in the physiological significance of gallium was aroused when it was noted that this element is contained in fission products (1). As a first approach to the problem the published methods for determining gallium in biological material (2, 3) were critically surveyed and tested in the laboratory. While these methods are useful in the range of 1 to 10 mgm. of gallium, it was necessary to develop an analytical procedure which could measure micro and semimicro quantities of this element.

The method herein described is based on the fluorescence of gallium 8-hydroxyquinolate in chloroform (4). This procedure has been applied to tissues of rats and rabbits which had been used in the toxicological and biochemical studies of gallium. These results appear to be the first published quantitative determinations of the distribution of gallium in the animal organism. Earlier qualitative spectrographic studies have been reported (5), which show that gallium is concentrated to some degree in the liver, spleen and kidneys.

Method. To a 500 cc. Kjeldahl flask add 25 cc. of concentrated sulfuric acid if the sample is 5 gm. or less. For 5 to 10 gm. of tissue add 50 cc. of sulfuric acid, and using 0.2 gm. selenium catalyst, digest to a pale straw color, cool and add 25 cc. of water. Add carefully solid sodium hydroxide pellets, finally making the solution slightly alkaline with dilute sodium hydroxide, using neutral red indicator. Limit the water added so that some solid sodium sulfate remains at this point. Add an additional three drops of neutral red indicator, and 5 cc. of 5 per cent 8-hydroxyquinoline (aqueous) solution. Add acid until a grey to greenish cloudy precipitate is formed. When tested with phenolphthalein, the solution should give a colorless reaction. By this means the pH is adjusted between 6 and 8, the optimum range for the separation of gallium.

Extract the mixture in a 500 cc. separatory funnel using two 50 cc. portions of redistilled chloroform. Combine the extracts in a 150 cc. pyrex beaker and add about 1 gm. powdered sodium bicarbonate to facilitate solution later, then evaporate to dryness on a steam bath. Place in a cool muffle furnace and heat to 650°C. for 1 hour. After cooling add a few cc. of water and make very slightly acid with dilute hydrochloric acid.

Photofluorometric determination. To a 50 cc. separatory funnel add two drops of thymol blue and 2 cc. of 20 per cent hydroxylamine hydrochloride solution; transfer the ashed extract to the funnel with a minimum of water so that the volume is not more than 35 cc. Allow to stand ten minutes to permit the reduction of Fe^{+++} to Fe^{++} but not more than 20 minutes to prevent atmospheric re-oxidation. Adjust the pH between 2.5 and 2.8. It is emphasized that the success of the method and its selectivity is largely based on the control of the pH at this point. To the flask add 2 cc. of 5 per cent 8-hydroxyquinoline solution which has been *adjusted to pH 2.5*. Extract the solution three times with 3 cc. portions of chloroform, combining the extracts to 10 cc. Cover the chloroform layer with

[1] The opinions or conclusions contained in this report are those of the author. They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department.

about 0.5 cm. of the aqueous phase to prevent atmospheric oxidation and evaporation and allow to stand at least 15 minutes before making the fluorometric reading. With the aqueous layer over the chloroform these tubes show no significant change in fluorescence in six hours. Read in a photofluorometer using filters for the emergent light which have a transmission peak at 635 mμ. Should the fluorescence be too intense dilute the chloroform solution in a ratio of ten to one and again make the photofluorometer readings.

Preparation of standards and reference curves. Because sodium salts are carried into the final extraction and since small amounts of other metallic ions also tend to reduce the

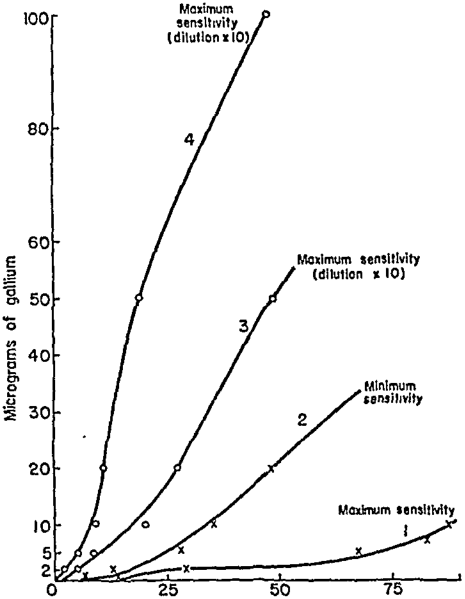


FIG. 1. PHOTOFLUOROMETER READINGS
Curves obtained when known amounts of gallium are added to 5 grams of tissue.

fluorescence of gallium, it is necessary to prepare reference curves using tissue blanks with added amounts of gallium.

To prepare the working standards, use 5 gm. of tissue, 25 cc. of concentrated sulfuric acid and add varying amounts of gallium. Also prepare a reagent blank with 5 gm. of tissue with no gallium added and carry through the digestion and extraction as outlined above. Plot the values obtained, galvanometer readings vs. microgm. of gallium, as shown in figure 1. These curves are used to estimate the amounts of gallium in unknowns, as this procedure applies corrections for such errors as are inherent in the method. Curves 3 and 4 in figure 1, were obtained by diluting the chloroform extract in a ratio of 10 to 1, and again making a photofluorometric reading. Concentrated solutions of the Ga-quinolate

materials which fluoresce in the ultra-violet, and this was the reason for preparing the sample by Kjeldahl digestion. Nitric acid and peroxide ashing procedures gave organic residues which produced an overwhelming fluorescence in the chloroform extract at all practical pH ranges. Dry ashing of tissues produced losses of added gallium up to 50 per cent due to volatility of the suboxide at 800–900° C.

The only interfering metals which produce fluorescence at pH 2.5–2.8 are indium and scandium. Both these elements produce negligible fluorescence (4) and have not been found by us in any biological materials. The photofluorometric determination of gallium in the presence of iron and copper makes necessary the rigid control of the pH at 2.5–2.8 in the final extraction. No further separation of iron is necessary for biological materials, providing the small amounts of Fe^{+++} are reduced to Fe^{++} by the hydroxylamine.

A silicone-type lubricant was used to prevent the solution in the chloroform of fluorescent substances often found in the more common stop-cock greases.

Examination of a variety of normal tissues, has shown that gallium is not a normal constituent. For bone the evidence is less conclusive. From our results it appears that traces of gallium may be present in bone but in amounts of less than 1 ppm. This amount is below the quantitative threshold of the analytical method described.

SUMMARY

A procedure is presented for the estimation of gallium in biological material, useful in the range of 2 to 100 microgm. Ga in 10 gm. or less of sample. Chloroform extraction of Kjeldahl digestates after neutralization and treatment with 8-hydroxyquinoline offers a means of separating micro quantities of gallium and other metals from biological samples.

Gallium initially localized in the kidney and bone, with lesser amounts in the spleen, liver, brain and muscle. The bones act as storage depots and yield gallium slowly, where appreciable quantities still persist for as long as 60 days after a single injection of gallium lactate.

REFERENCES

1. Plutonium Project: J. Am. Chem. Soc., 68: 2411, 1946.
2. Mellan, I.: Organic Reagents in Inorganic Analysis. The Blakiston Company, Philadelphia, 1941.
3. Sandell, E. B.: Colorimetric Determination of Traces of Metals. Interscience Publishing Co., Inc., New York, 1944.
4. Sandell, E. B.: Anal. Chemistry, 19: 63, 1947.
5. Levaditi, C.: Compt. rend. Acad. d. Sci., 193: 117, 1931.
6. Robinson, W. O., Dudley, H. C., Williams, K. T., and Byers, H. G.: Ind. Eng. Chem., Anal. ed., 6: 274, 1934.
7. Dudley, H. C., and Garzoli, R. F.: J. Am. Chem. Soc., 70: 3942, 1948.

STUDIES OF THE TOXIC ACTION OF GALLIUM[1]

H C DUDLEY AND MILTON D LEVINE

Naval Medical Research Institute, National Naval Medical Center, Bethesda 14, Maryland

Received for publication December 15, 1948

As a result of the recent interest in gallium and some of its compounds (1), a comprehensive study of the physiological and pharmacological activity of gallium has been undertaken Further interest in the action of radio gallium has also been aroused by the preliminary findings of this study

Since a survey of the literature (2–6) yielded such a small amount of useful information, a study of the toxic dosages (LD_{50}), pathology, influence of mode of administration, etc , were of first consideration The results are presented here of a study of the toxic action of gallium through (1) inhalation of an aerosol of gallium chloride, (2) ingestion of diets containing the chloride or gallium lactate, and (3) injection of gallium lactate, both intravenously and subcutaneously.

EXPERIMENTAL *In vitro* observation in this laboratory has shown that both gallium chloride and nitrate, as well as the alum complex, $NH_4Ga(SO_4)_2$, are efficient protein precipitants, and that the chloride and nitrate were almost completely and rapidly precipitated as the gelatinous hydroxide at pH 7 4 This protein precipitating action and formation of the insoluble hydroxide at tissue pH probably accounts for the marked localized reaction resulting at the site of injection Obviously, these salts are unsatisfactory for injection For the present work, gallium lactate (7) was chosen and prepared for the injection experiments since lactic acid may be considered nontoxic (5), certainly in the quantities administered in these studies

Since the publication of the report dealing with gallium lactate as a suitable medium for the administration of gallium (7) it has been found that purified gallium lactate brought to a pH 7 0 by the addition of dilute ammonia may, under certain circumstances, produce a faintly opalescent colloidal solution This colloidal solution has caused immediate death when injected intravenously into five rabbits, due to the formation of pulmonary and/or coronary emboli This solution when administered subcutaneously is absorbed slowly and produces no rapid deaths and is much less toxic on a mgm /kgm basis than a true solution of gallium lactate

We have prepared a satisfactory solution for most types of pharmacological studies of gallium by adding a small amount of sodium lactate to the gallium lactate (solution 5 mgm Ga/cc plus 5 mgm Na lactate/cc) thus stabilizing the gallium and preventing the formation of a colloidal hydroxide The pH is brought to about 6 0 (brom cresol purple indicator) by adding powdered sodium bicarbonate After heating to boiling, this solution should be clear, at pH 6 5 to 7 0, and if no cloudiness has appeared, it is stable to autoclaving and suitable for intravenous or other routes of administration

Exposure of rats to aerosols of gallium chloride A device consisting of a nebulizer, exposure chamber and trap for recovering the gallium was made of glass With this apparatus an aerosol of uniform particle size was created and the chamber was kept at atmospheric

[1] The opinions or conclusions contained in this report are those of the authors They are not to be construed as necessarily reflecting the views or the endorsement of the Navy Department

pressure while a single animal was exposed to a constant concentration of the compound. White rats weighing between 150 and 250 gm. were used.

Gallium chloride solutions were prepared in concentrations ranging from 18.4 to 94.4 mgm. of gallium per cc. The pH of these solutions was adjusted to 2.2, the highest pH at which the gallium chloride will remain in solution. Control solutions of sodium chloride were made equimolar to the gallium chloride solutions and adjusted to a pH 2.2. The rats were exposed to the aerosols of the nebulized solutions for periods varying from 30 to 240 minutes.

To determine the gallium concentration of the air in the chamber at any given time, a known volume of air from the chamber containing the aerosol was drawn through a sintered glass funnel, covered with a damp asbestos mat. The funnel was washed with water and the wash solution was analyzed gravimetrically for gallium by precipitating as the 8-hydroxyquinoline complex.

Nine animals were exposed for periods of one-half to four hours to atmospheres containing an aerosol of gallium chloride. Exposures were to concentrations of 0.025 to 0.125 mgm. Ga/liter of air. Of these rats all survived but one, which died during exposure to the maximum concentration, after three hours. Of five control rats exposed to sodium chloride aerosols, two died during exposure after two to three hours.

Animals exposed to both the aerosols, were found to have bilateral areas of consolidation of the lungs with some punctate hemorrhages. Since the gallium chloride solution was at pH 2.2, the sodium chloride solution was similarly adjusted. This acidity accounts for the pathological condition seen in these animals and is responsible for the deaths. Analysis of the tissue from animals so exposed to gallium chloride shows that no gallium passed the lung tissue but was deposited in the alveoli, probably as the hydroxide. Therefore, studies of this series of animals were not extended.

Feeding experiments. Four series of young rats (initial weights 105–140 gm.) were fed for 13 weeks on diets containing gallium chloride in concentrations of 10, 100, 500 and 1,000 parts gallium per million of feed. Each series consisted of 10 rats, with pair-fed controls

There was no evidence that the diets containing gallium produced any effect whatsoever. The animals' conditions were excellent throughout the test period and the weight curves paralleled the control curves. The average weight gain of the 10 rats receiving 1 gm. gallium/kgm. of food (1000 p.p.m.) was 23.6 per cent, while the pair-fed controls averaged 23.5 per cent weight gain during the 13 weeks of observation. There were no deaths during this period in the 40 rats receiving gallium chloride or the 20 pair-fed controls.

Chemical examination of the tissues of the animals showed no detectable amounts of gallium present in the liver, spleen or kidney of the animals after the completion of the 13 weeks feedings. The bone contained trace amounts. These findings show that in rats there is little or no absorption of gallium chloride from the intestinal tract, although the average daily intake may be as high as 20 mgm. Ga per animal. The alkalinity of the intestinal tract is sufficient to convert gallium chloride to the hydroxide or other insoluble complex.

In order to provide a salt stable at the pH of the intestinal tract, gallium lactate was incorporated in the diet of rats since this salt is stable at the pH of 7 0–7 6 Two series of young rats (initial weight 88 to 112 gm), were fed for 26 weeks on diets containing gallium lactate in concentrations of 500 and 1000 parts gallium per million of feed Each series consisted of 20 rats, 10 males and 10 females, with an equal number of pair fed controls These groups were fed on sufficient commercially prepared diet to yield normal growth curves

The average weight increase of the 20 rats receiving 1 0 gm Ga/kgm of food (1000 p p m) was 167 per cent while the pair fed controls gained 192 per cent in weight during the same period The 20 rats receiving the 0 5 gm Ga/kgm. of food (500 p p m) gained 177 per cent in weight during the 26 weeks feeding; controls also showed 192 per cent gain in weight No other effects were noted in these animals, other than the slight difference in the average weight curves There was but one death in the animals being fed the gallium lactate (1 female on 500 p p m) and one death in the control males The general condition of the entire colony was excellent throughout the test

Chemical examination of the tissues of the animals fed gallium lactate at a level of 15 to 20 mgm Ga per day (1000 p p m) showed that there were trace amounts of gallium in the liver, spleen and kidney, while the bone contained amounts from one to three microgm /gm of tissue (1 to 3 p p m) These findings show that gallium in a form soluble at pH 7 6 is not readily absorbed from the intestinal tract Therefore, in general, gallium compounds offer little hazard from the standpoint of an ingested poison It also appears likely that the small portions that do reach the circulation are readily excreted since the bone has been shown to concentrate injected gallium extensively and yield it slowly to the general circulation

Injection studies In order to study the acute toxic effect of gallium, uncomplicated by other possibly toxic molecular compounds, rats and rabbits were injected with a single dose of purified gallium lactate The lactate was dissolved in distilled water at a concentration of 5 mgm Ga/cc Sodium lactate solution was added so that the final concentration of this salt would also be 5 mgm /cc Powdered sodium bicarbonate was used to adjust the pH to 6 0 and the solution autoclaved Concentrations of gallium lactate in excess of 5 mgm Ga/cc have caused localized reaction at the site of injection

Normal, healthy albino rats weighing from 105 to 220 gm were injected intravenously in the large tail vein, the solution was administered to normal adult rabbits through one of the large ear veins In both rats and rabbits subcutaneous dosage was given on the broad portion of the back The animals were then housed in clean, well ventilated animal cages and observed closely for a period of 30 days or longer

Controls for the above series of injection studies were carried out by injecting a solution containing 48 2 mgm sodium lactate per cc (pH 7 0) which was equimolar to a solution of gallium lactate containing 10 mgm Ga/cc In each case the controls received sodium lactate equivalent to the largest dose of gallium lactate administered Results of the control studies are shown in tables 1 and 2

On death, animals were autopsied if marked autolysis had not occurred The gross pathological findings were recorded and representative tissues were taken for sectioning and microscopic examination Details of these findings will be reported later

In the rat injection experiment, 35 rats were used for intravenous studies Animals which received 28 to 38 mgm Ga/kgm showed hyperexcitability the

first 24 hours following injection, then developed a semistuporous attitude, with limited activity and marked drop in weight and food intake. Rats receiving 40 to 50 mgm. Ga/kgm. were quickly affected and 40 to 60 per cent died within 24 to 72 hours after injection. Of the 15 animals observed when moribund, all showed a flaccid paralysis of the hind limbs. Details of these findings are summarized in table 1.

TABLE 1

Acute toxicity of gallium lactate administered to rats

NO. OF RATS TREATED	DOSAGE MGM. Ga/KGM. BODY WT.	DEAD IN 10 DAYS	REMARKS
Intravenous injection			
		per cent	
10	20–38	0	Marked loss in weight; poor condition
5	42	40	All deaths within 3 days. Survivors lost weight. Poor condition for 30 days
5	47	60	
5	51	60	
10	57–75	100	All deaths in 5 days
5	Controls 360 mgm. Na lactate/kgm. No observable effect		
Subcutaneous injection			
30	20–50	0	No symptoms except transitory loss in weight
10	90	10	Survivors showed neurological symptoms, including blindness and photophobia for 5 to 10 days. Full recovery occurred 30 to 45 days following injection
10	100	10	
10	110	40	
20	120	50	
10	130	50	
20	140	80	
20	150	75	
10	160	100	All dead in 5 days
10	170	100	All dead in 5 days
20	180	100	All dead in 2 days
5	Controls 865 mgm. Na lactate/kgm. No observable effect		

NOTE: On the basis of these data, the LD$_{50}$ (10 days) of gallium as gallium lactate, is calculated by probit analysis to be for the rat:

LD$_{50}$ (intravenous).. 46 mgm. Ga/kgm.

LD$_{50}$ (subcutaneous)..................................... 121 mgm. Ga/kgm.

Eleven animals were autopsied immediately after death. Gross examination showed the kidneys to be extremely pale in each case. Six animals had punctate hemorrhages in the cortices, one animal showed frank hemorrhage into both pelves.

The blood urea nitrogen (B.U.N.) of another series of 25 rats was studied.

Representative results are presented in table 2 for animals given intravenously sufficient gallium lactate to insure death in a few days.

In those animals dying within 24 hours following injection of gallium lactate the B.U.N. was within normal limits or only slightly elevated. However, the rats which died 24 to 72 hours following injection showed marked elevation of the B.U.N. On microscopic examination of the kidneys from the rats injected with the lactate, early granular hyalin changes in the tubules were observed. Occasional swollen glomeruli showing increased segmented cells suggested inflammatory change.

TABLE 2

Blood urea nitrogen of rats injected with gallium lactate

ANIMAL NO.	DOSAGE INTRAVENOUS INJECTION	GROSS KIDNEY PATHOLOGY	TIME INTERVAL INJECTION TO DEATH	B.U.N.
	mgm. Ga/kgm.		*hrs.*	*mgm. %*
34	95	Cortex pale	4	19
33	102	Multiple hemorrhages of cortex	5	41
35	100	Light in color	7	29
32	65	Cortex extremely pale	24	79
36	95	Multiple hemorrhages of cortex	72	223
Controls given Na lactate intravenously				
38	Sodium lactate equimolar to gallium lactate 100 mgm./kgm.		killed	10
39			killed	16
40			killed	24
41			killed	23

In 16 normal untreated rats the B.U.N. ranged between 8.9 and 25.6 mgm. % with an average of 15.8 mgm. %.

These results suggest that animals succumbing to gallium poisoning within 24 hours do not die a renal death. However, for those surviving for more than 48 hours the evidence is suggestive that renal pathology results.

On the basis of the data presented above and in table 1, it is concluded that the dose of gallium as gallium lactate required to kill within ten days 50 per cent of rats injected intravenously is 46 mgm. Ga/kgm. (LD_{50}, 10 days = 46 mgm. Ga/kgm.).

Gallium lactate was injected subcutaneously into 170 rats. At a level of 20 to 50 mgm. Ga/kgm. there was no observable effect other than a transitory weight loss and a tendency toward hyperexcitability lasting for only 24 hours. At levels of 100 to 180 mgm. Ga/kgm. the animals showed a marked excitability for 24 to 48 hours and then exhibited a characteristic torpid attitude, bordering on semi-coma, although they were conscious. Some evidence of photophobia was evident for up to 30 days. At these levels those animals which survived three to five days, developed a diarrhea, became emaciated and ate little food;

in many cases blindness developed two to four days prior to death. In certain of these animals held for observation up to six months after injection the reduction in activity and in food intake lasted for 30 to 45 days, beyond which increases in weight and activity followed so that at 60 days, all of the survivors were in good condition (see table 1). All animals surviving the first ten days

TABLE 3

Acute toxicity of gallium lactate administered to rabbits

NO. OF RABBITS TREATED	DOSAGE MGM. Ga/KGM. BODY WT.	DEAD IN 10 DAYS	REMARKS
Intravenous injection			
		per cent	
20	15–35	0	No observable effects other than transitory weight loss
5	40	0	Marked transitory effects
10	45	70	Death in 6 to 10 days
5	50	100	All deaths were within 5 days, and were accompanied by serous exudate from the nose. Death was due to respiratory failure
5	55	100	
5	60	100	
5	70	100	
5	Controls 335 mgm. Na lactate/kgm. No observable effect other than transitory weight loss		
Subcutaneous injection			
5	70	0	Slight transitory loss in weight
5	80	0	Marked transitory loss in weight
5	90	40	Deaths resulted from acute respiratory failure. Survivors showed marked loss in weight and reduced activity. Some survivors showed some delayed toxic effects 30 days following injection. At 60 days all survivors were normal
10	100	60	
5	110	80	
5	120	100	
5	130	100	
5	Controls 575 mgm. Na lactate/kgm. No observable effects		

NOTE: On the basis of these data the LD_{50} (10 days) of gallium, as gallium lactate, is calculated by probit analysis to be for the rabbit:

LD_{50} (intravenous) .... 43 mgm. Ga/kgm.
LD_{50} (subcutaneous) .... 98 mgm. Ga/kgm.

eventually recovered from the effects of the one subcutaneous dose of gallium.

On the basis of the data presented in table 1, it is concluded that the quantity of gallium as gal ium lactate required to kill within ten days 50 per cent of the rats injected subcutaneously is 121 mgm. Ga/kgm. (LD_{50}, 10 days = 121 mgm. Ga/kgm.).

In table 3 are shown the results of the injection of gallium lactate into rabbits which had been kept under observation for at least two weeks prior to the be-

ginning of the experiment. These data indicate that intravenous dosages in excess of 43 mgm. Ga/kgm. will produce more than 50 per cent deaths, usually within 72 hours, while a dose of 97 mgm. Ga/kgm. is required to produce 50 per cent deaths within ten days when the lactate is administered subcutaneously. Such deaths, seemingly from respiratory failure, were preceded by a flaccid paralysis, particularly in the hind limbs. No other particular neurological symptoms were observed. Gross autopsy findings were essentially negative for all organs except the lungs which were often edematous and hemorrhagic.

SUMMARY AND CONCLUSIONS

Rats exposed to aerosols of gallium chloride were affected but slightly in concentrations of 0.125 mgm. Ga/liter of air in exposures of from ½ to 4 hours. The acidity of the solution (pH 2.2) of both gallium chloride and the sodium chloride controls produced the effects noted. Gallium is deposited in the lungs as the insoluble hydroxide.

Rats fed gallium chloride or gallium lactate in levels up to 1000 p.p.m. (1 gm. Ga/kgm. food) showed no significant effect in periods up to 26 weeks. Absorption of gallium salts from the intestinal tract is negligible.

The minimum lethal doses (LD_{50}) of gallium lactate for rats and rabbits are as follows:

Rats.	Intravenous injection	47	mgm.	Ga/kgm.
	Subcutaneous injection	121	"	"
Rabbits.	Intravenous injection	43	"	"
	Subcutaneous injection	97	"	"

The symptoms resulting from lethal and near-lethal injections of gallium salts may include rapid loss in weight, hyperexcitability, and in some cases photophobia, blindness and terminal flaccid paralysis. Early pathological findings indicate death may be due to acute respiratory failure, with some kidney pathology resulting 3 to 4 days after injection. In general, animals surviving 10 days after a near fatal dose of injected gallium, recover within 30 to 45 days.

REFERENCES

1. Plutonium Project: J. Am. Chem. Soc., 68: 2411, 1946.
2. Rabuteau, M.: Compt. rend. Soc. de Biol., Ser. 7, 5: 310, 1883.
3. Schwarz, L., and Sicke, F.: Arch. Hyg., 100: 143, 1928.
4. Levaditi, C.: Compt. rend. Acad. d. Sci., 193: 117, 1931.
5. Sollman, T.: Manual of Pharmacology, 7th ed., Saunders, Philadelphia, 1948.
6. Meek, S. F., Harrold, G. C., and McCord, C. P.: Indust. Med., 12: 7, 1943.
7. Dudley, H. C., and Garzoli, R. F.: J. Am. Chem. Soc., 70, 3942, 1948.

THE DISTRIBUTION OF RADIOACTIVITY IN RATS AFTER ADMINISTRATION OF C^{14}-LABELED METHADONE[1]

HENRY W. ELLIOTT, FRANCES N-H. CHANG, ISMAIL A. ABDOU, AND HAMILTON H. ANDERSON

The Division of Pharmacology and Experimental Therapeutics, University of California Medical School, San Francisco 22, California

Received for publication December 18, 1948

The determination of morphine-like analgetics in tissues has been difficult because of the dilution in the body of the small doses of drug required to produce analgesia. For example, 10 mgm. of methadone is diluted to approximately one part in seven million in a 70-kilo man, if uniform distribution is assumed. Correspondingly high dilutions may be calculated for other agents, such as morphine and meperidine. However, colorimetric methods have been reported for the determination of these agents in tissues or body fluids where they may concentrate. Gauss (1) was able to recover morphine quantitatively from muscle or liver at a dilution of 1:1,000,000. Way *et al.* (2) determined meperidine in rat blood at a dilution of 1:50,000. Cronheim and Ware (3) detected methadone at a dilution of 1:2,000,000.

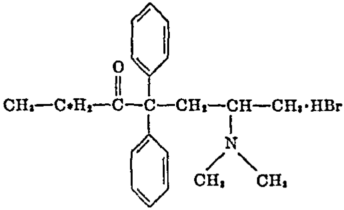
In spite of the development of these methods they have not led to comprehensive studies on the distribution and fate of these compounds probably because of the difficulties involved in the extraction of minute quantities of drug from relatively large amounts of tissue. So little is known regarding the mechanism of action of analgetics on the cellular or even organ level that distribution studies may well be helpful as a first step in elucidating their mode of action.

The recent availability of long-lived radioactive carbon has made feasible the synthesis of labeled analgetics which can be followed through the body by standard tracer techniques. By sacrificing animals at intervals after administration of the radioactive drug and measuring the radioactivity present in the various organs, distribution may be correlated with systemic effect. Similarly, the rate of absorption from subcutaneous or intramuscular sites of injection may be determined and excretion studied by radioactivity measurements of excreta and of the organs of excretion long after the pharmacologic effects of the drug have disappeared. If the drug passes through the body unchanged or is conjugated, tissue concentrations may be calculated directly from radioactivity measurements. If the molecule labeled with C^{14} breaks up into fragments which can be oxidized, radioactivity may be detected in the carbon dioxide of the expired air. When the drug is known to break down in the body, care must be used in the interpretation of tissue radioactivity measurements, since one cannot be sure that the measured activity represents the intact molelule and not merely a portion thereof.

[1] Aided by a grant from the National Institutes of Health, Bethesda 14, Maryland.

The experiments to be described in this report consist of studies on the absorption, distribution, and excretion of methadone labeled with C^{14}. This compound was chosen for study because of its possible importance as a morphine substitute and because it is thought to be partially excreted unchanged in the urine (3).

METHODS. The synthesis of the labeled methadone used for our experiments was accomplished by Chang and Sah in this laboratory in association with chemists of the Radiation Laboratory, Berkeley Campus, and has been described elsewhere by Tolbert *et al.* (4). Briefly, the method consisted of condensation, using the Grignard reaction, of radioactive ethyl bromide ($CH_3C^*H_2Br$) and methadone nitrile to give methadone containing a radioactive carbon atom in the 2-position. The product has a specific activity of 0.547 μc/mgm.



Radioactive 6-dimethylamino-4-diphenyl-3-heptanone hydrobromide

It was felt that the presence of C^{14} in the 2-position would aid in determining whether or not methadone was broken down in the body since cleavage at the 2- or 3-position would result in products very likely to be oxidized to carbon dioxide and water. If such were the case, radioactivity would be present in the expired air.

This preparation when compared with a commercial non-radioactive product (Dolophine, Lilly)[2] produced equivalent biologic response in rats. Ten mgm./kgm. produced analgesia which persisted for at least 3 hours, accompanied by loss of righting reflex for 2 hours, postural rigidity, respiratory depression and the Straub reaction.

Six young adult female rats of the Slonaker-Wistar strain weighing between 106–158 gm. and maintained on a diet of Purina Dog Chow and tap water were used in these studies. Each was given 10 mgm./kgm. of labeled methadone by subcutaneous injection into the left hind leg. According to Finnegan *et al.* (5), this dose is one-tenth of the LD_{50} for methadone administered to rats by this route. Immediately after injection each animal was placed in a glass metabolism cage equipped with an absorber for exhaled CO_2. After 1, 2, 3, or 24 hours, the animals were removed from the cage and prepared for dissection. Ether was administered if necessary, and in each case, just before sacrifice, a blood sample was obtained from the abdominal aorta. Any urine in the bladder was removed with a syringe, and the organs were dissected out for radioactivity measurements. In some cases the intestines and stomach were washed out with isotonic saline solution.

The isolated tissues including the injection site, the skin and hair, and the carcass were dried in weighed porcelain dishes at 60°C. for 48 hours and ground to a fine powder after determining dry weights. Radioactivity was measured essentially by the method described by Dauben, Reid, and Yankwich (6). Tissue combustion was carried out in an analytical type combustion train; the CO_2 evolved was absorbed in sodium hydroxide. Small tissues were burned in entirety, but when the dry weight of an organ exceeded 200 mgm. it was

---

[2] Supplied through the courtesy of Dr. E. C. Kleiderer, Lilly Research Laboratories, Indianapolis 6, Indiana.

necessary to take an aliquot for combustion. Ordinarily, samples of 100–150 mgm. were used in such cases. Barium carbonate was precipitated from the sodium carbonate solution obtained by combustion, collected on sintered glass funnels and weighed. Counting was done on aluminum discs under a bell counter with a thin mica window.

The errors involved were those of ordinary analytical procedures plus the statistical errors of counting. The analytical error was determined by burning three 0.008 mgm. samples of methadone* giving an average value of 1674 $\pm$ 46 counts per minute or 2.8 per cent. From this datum and the known specific activity of the methadone* (0.547 μc/mgm.) the counter efficiency was found to be 17 per cent. Each sample was counted for a maximum of 30 minutes which allowed about 15 per cent error for samples which assayed 10 counts per minute or less above background. However, the tissues which contained most of the activity were counted for a sufficient time to make the error 5 per cent or less. The seemingly large errors calculated for counts from tissues of low specific activity apparently had little effect on the final balance, since in 5 out of the 6 rats recovery of the administered dose was 100 $\pm$ 4 per cent. In order to improve appreciably the counting accuracy of samples of low specific activity, 8-hour counts would have been required, and the expenditure of such a great amount of time was not considered advisable in view of the small contribution of these samples to total activity.

RESULTS. *Possible Integrity of the Methadone* Molecule.* In biologic studies in which a complex organic molecule labeled with radioactive carbon is administered to an animal, it is often difficult to determine whether or not the radioactivity measured in tissues is present as the intact compound. In the present instance, justification for assuming an equivalence between specific activity and methadone* concentration is provided by the following evidence.

Collection in sodium hydroxide of the expired air from rats treated with methadone* for periods up to 3 hours yielded no radioactivity. Since methadone* was synthesized with the C^{14} atom in the 2-position, it is apparent that the molecule is not broken in the 2- or 3-position to yield compounds which can be oxidized in the body to CO_2 and H_2O. When compounds containing C^{14} and known to be oxidized in the body are administered to animals, activity is eliminated through the lungs. For example, Reid and Jones (7) found activity in the expired air of mice after giving the animals radioactive tyrosine.

Further evidence that the activity measured in all experiments was present as methadone*, either intact or in conjugated form, was obtained by study of the distribution of activity in one rat 24 hours after methadone* administration. Ninety-eight per cent of the activity recovered was in the excreta or intestines. Such complete excretion suggests that the methadone* molecule is handled by the body as a unit; at least, there is no evidence that a radioactive fragment is left behind.

Finally, isotope dilution studies were done on the bile of 3 rats not included in this series. The bile duct was cannulated and methadone* was administered in the usual manner. A portion of the bile obtained over a 2-hour period after drug administration was combusted and found to be radioactive. One hundred mgm. of commercial methadone HCl was added to the remainder after acidification, and the free base was precipitated as an oil from alkaline solution. This material crystallized with difficulty overnight at 4°C. and was separated by centrifugation. The supernatant fluid was somewhat cloudy and may have contained a

small amount of uncrystallized methadone. Analysis showed but a fraction of the original activity present in the supernatant fluid. This would be the expected result if the activity were originally present in the bile as methadone* which would precipitate along with the added carrier methadone. Isotope dilution work is continuing to determine the exact chemical combination of the C^{14} in the animal.

The available evidence seems to indicate that the activity measured in these experiments was present in the animal body as methadone* either free or conjugated and not as a fragment of the molecule. Therefore, for the sake of simplicity, specific activity values have been converted to microgm. methadone* per gm. dry tissue.

Absorption and Blood Levels. To determine the rate of absorption of methadone* the injection site (left hind leg) of each rat was separated from the carcass and its specific activity measured. Assuming specific activity to be proportional

TABLE I

The absorption of methadone after subcutaneous injection of 10 mgm. per kgm. in the left hind leg*

	RAT NUMBER					
	1	5	3	4	2	6
	Hours after injection					
	1	1	2	2	3	24
* Inj. site conc. (A)	218	181	56	71	49	0
* Carcass conc. (B)	10	17	20	15	12	0
A − B	208	164	36	56	37	0
* Blood conc.	5	6	6	5	16	0

* Concentrations expressed as microgm. methadone* per gm. dry tissue.

to concentration, activity values were converted to microgm. of methadone* per gm. of dry tissue. In table I, injection site and carcass concentrations are compared. The difference may be interpreted as a rough measure of absorption. It is apparent that absorption is incomplete after 1 hour, but equilibrium is reached in 2 hours or less.

Concomitant blood concentrations also listed in table I indicate that methadone* is distributed throughout the body via the circulatory system.

Distribution. The distribution of methadone* in various organs and tissues is summarized in table II. This table is so arranged that in all 6 animals tissue concentrations and per cent of activity recovered can be compared at 1, 2, 3, and 24 hours In general, the values for tissues containing 3 per cent or more of the recovered activity are accurate to 5 per cent or better. The values for tissues of low specific activity may be in error by as much as 15 per cent, as explained above.

The degree of concentration in an organ may be determined by comparing the value for the organ with that of the carcass. Examination of table II shows that, in spite of the marked effects of methadone on the central nervous system,

there was no appreciable concentration of the drug in the brain even at the one-hour period when analgesia was pronounced. In fact, with the exception of rat 1, the concentration values for brain tissue were lower than those for the carcass.

TABLE II

The distribution of methadone in rats at intervals after injection of 10 mgm. per kgm.*

	RAT NUMBER											
	1		5		3		4		2		6	
Time in hours	1		1		2		2		3		24	
Recovery	97.1%		83.5%		102.2%		103.5%		103.2%		104.0%	
	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%	Conc.	%
Brain stem	63	0.5	13	0.1	13	0.1	2	0.01	0.3	0.001	—	—
Cerebellum	59	0.2	10	0.03	11	0.03	4	0.01	0.2	0.001	—	—
Cerebrum	33	0.5	11	0.2	13	0.2	6	0.1	12	0.2	—	—
Liver	48	6.0	82	11.2	70	7.1	72	9.2	46	5.6	11	1.3
Stomach and contents	64	2.5	48	2.6	238	6.0	80	8.6	189	11.1	2	0.04
Small intestine and contents	92	12.6	50	8.1	140	16.1	95	15.4	349	39.3	52	4.1
Large intestine and contents	9	1.0	22	1.9	31	2.7	30	3.3	21	1.9	79	6.1
Kidney	60	1.3	101	2.2	63	1.3	74	1.7	24	0.5	2	0.06
Lungs	139	1.6	222	4.1	192	3.2	191	2.9	124	2.0	1	0.02
Spleen	61	0.7	31	0.3	45	0.7	45	1.0	43	0.6	2	0.03
Adrenals	285	0.5	95	0.13	51	0.08	61	0.06	34	0.05	—	—
Thyroid	—	—	110	0.03	64	0.03	61	0.02	—	—	—	—
Uterus and ovaries	36	0.3	18	0.2	15	0.2	14	0.1	13	0.1	—	—
Mesentery and abdominal fat	16	1.4	10	1.4	18	3.8	19	2.0	8	1.1	—	—
Blood	5	0.5	6	0.9	5	0.4	6	0.4	16	0.8	0	0
Heart	30	0.3	50	0.4	32	0.3	25	0.2	0.1	0.001	—	—
Muscle	20	1.0	26	2.0	22	1.5	15	1.2	11	0.8	—	—
Skin and hair	14	10.6	4	2.3	20	16.4	16	9.9	3	2.0	0	0
Carcass	10	17.2	17	28.7	15	25.6	20	29.9	12	20.5	0	0
Left hind leg (injection site)	218	41.1	181	32.5	71	12.8	56	10.3	49	11.0	0	0
Excreta	—	—	(−)	0.7	(−)	1.5	(−)	3.5	(−)	2.3	(−)	88.3

Conc. = microgms methadone*/gm. dry tissue.

$$\text{Recovery} = \frac{\text{counts recovered}}{\text{counts injected}}.$$

% = per cent of total activity recovered.

(−) = concentrations not calculated.

The tissues which contained significant quantities of methadone were chiefly the organs of excretion: the liver, gastro-intestinal tract and kidneys. The lungs and spleen also contained concentrations well above the carcass level. These organs retained some activity for 24 hours in contrast to tissues, such as muscle, which were completely devoid of activity after 24 hours. The gastro-intestinal ac-

tivity increased with time suggesting the importance of this route for methadone excretion.

Finally, high concentrations of activity were found in the adrenals. Although this may be related to vascularity since the thyroid shows a similar effect, the adrenal concentration is high during the period of maximum drug action and decreases as the animal recovers. This aspect of the concentration-action of methadone deserves further study.

Excretion. An evaluation was made of the activity measurements in the excreta and organs of excretion during the period of peak drug activity and at 24 hours to determine the routes of methadone* excretion. No radioactivity was found in the expired air during the first 3 hours after methadone* administration,

TABLE III

Per cent of total activity recovered in excreta and organs of excretion after methadone administration*

	RAT NUMBER					
	1	5	3	4	2	6
	Hours after injection					
	1	1	2	2	3	24
Liver	6.0	11.2	7.1	9.2	5.6	1.3
Small intestines	12.6	5.8	16.1	11 3	39.3	0.6
Contents of small intestines	—	2.3	—	4.0	—	3.5
Total small intestine	12.6	8.1	16.1	15.3	39.3	4.1
Colon and contents	1.0	1.9	2.7	3.3	1.9	6.1
Feces	—	—	—	—	—	58.4
Kidneys	1.3	2.2	1.3	1.7	0.5	0.1
Urine	—	0.7	1.5	3.5	2.3	30.0[a]

[a] Includes cage washings.

so it appears unlikely that the molecule is broken up into fragments capable of oxidation to carbon dioxide and water.

In the course of the distribution studies it was soon apparent that methadone* was rapidly localized in the gastro-intestinal tract. High concentrations were found in the small intestines of rats 1–3. Analysis of a portion of the duodenal contents of rat 4 showed that 27 per cent of the total intestinal activity was contained in the small amount of fluid expressed from the duodenal lumen. In rat 5, 28 per cent of the total activity present in the small intestine was removed by washing with 0.9 per cent saline solution. Twenty-four hours after methadone* administration approximately 70 per cent of the activity recovered was in the liver, intestines, and feces; most of the remainder was in urine or cage washings.

Renal excretion is appreciable although secondary to excretion by the intestinal route. Urine passed during the experimental period or collected from the bladder before dissection was highly active. Urine and cage washings from rat 6, sacrificed at 24 hours assayed 30 per cent of the total recovered activity. In table III are presented the data pertinent to excretion.

DISCUSSION. The rate of absorption recorded is compatible with the observed effects of 10 mgm./kgm. of methadone administered subcutaneously to rats. Analgesia, central depression, and catatonia are most profound within 30–90 minutes, and recovery is well under way at 120 minutes. At 60 minutes only 30 per cent of the dose was found at the injection site, and at 120 minutes absorption was virtually complete. It may be concluded that methadone* HBr is rapidly and completely absorbed after subcutaneous administration.

The distribution studies indicate that methadone* is not concentrated to any degree by the central nervous system. The small quantities of activity found there may have resided in blood trapped in the brain at the time of sacrifice. Because of the profound effects of methadone on the central nervous system, it was surprising to find brain levels even lower than carcass concentrations. Either methadone exerts its effects on the brain at very low concentrations, or its site of action is outside the central nervous system altogether.

With the latter possibility in mind, the relatively high concentration in the adrenals of the one-hour rats is of interest, since methadone is known to increase blood sugar levels (5). In addition, recent reports implicate epinephrine as a mediator of the analgetic effect of various agents (8–10). Since the quantities of methadone* found in the brain were small, the existence of a "target organ" outside the central nervous system is entirely possible. The ability of methadone to inhibit cholinesterase (11) may be of importance in this connection.

The greatest quantities of methadone* were found in the liver and intestines even at 1 hour. Experiments in progress (12) indicate that methadone* passes from the liver to the small intestine via the bile duct, although the possibility of mucosal excretion, especially in the stomach, has not yet been excluded. In either case the concentrations attained in the small intestine are of sufficient magnitude to suggest that the effects of methadone on intestinal motility (13) could be due to a direct action on the organ or its local innervation.

Reabsorption from the intestinal lumen may occur, but such material should be quickly returned to the gut via the liver. Excretion predominates since the quantity in the small intestine increased over the 3-hour period studied. The importance of the gastro-intestinal tract as a route of excretion is emphasized by the fact that in the 24-hour animal 58 per cent of the dose was recovered in the feces.

Urinary excretion of methadone has been demonstrated by others (3, 14) using colorimetric methods. Our tracer studies also indicate excretion by the kidneys. Bladder urine from animals sacrificed at 3 hours or less contained appreciable amounts of activity and 30 per cent of the activity recovered from the 24-hour animal was in the urine or cage washings. The quantity actually excreted by the kidneys of this animal is probably less than 30 per cent due to contamination of the urine by feces, but it is apparent that both gastro-intestinal and urinary excretion must be considered in methadone balance studies.

The foregoing data indicate the need for further studies to determine the mechanism by which methadone produces analgesia. Unless it can be shown that the minute quantities found in the brain can account for the observed effects of the

drug, it must be assumed that analgesia is mediated peripherally. In this connection, the possible role of the adrenal medulla and the sympathetic nervous system in general is worthy of consideration.

The authors wish to thank Dr. B. M. Tolbert of the Radiation Laboratory, Berkeley Campus, and Drs. P. P. T. Sah and J. Oneto of the San Francisco Campus for their part in making the labeled methadone available to us. Dr. L. L. Eisenbrandt and Mrs. T. K. Adler gave valuable assistance with some of the analytical work and in preparation of the manuscript.

SUMMARY

1. Methadone* HBr labeled with C^{14} in the 2-position proved to be equally effective as an analgetic in rats (10 mgm./kgm.) as the unlabeled drug.

2. Tissue specific activity values appear to be comparable to methadone* concentrations. The possibility that methadone is conjugated in the body has not been completely excluded.

3. Methadone* HBr is rapidly and completely absorbed after administration by the subcutaneous route.

4. At periods of from 1 to 3 hours after administration, the specific activity of brain was no higher than carcass levels, thereby indicating no particular affinity of central nervous system tissue for methadone*. Concentrations well above carcass levels were found in the adrenals (at 1 hour), thyroid, lungs, spleen, kidneys, liver, and gastro-intestinal tract.

5. Methadone* HBr is almost completely excreted after 24 hours. Excretion is primarily via the intestinal route and secondarily via the kidneys.

REFERENCES

1. Gauss, H.: J. Lab. Clin. Med., 6: 699, 1920–21.
2. Way, E. L., Swanson, R., and Gimble, A. I.: This Journal, 91: 178, 1947.
3. Cronheim, G., and Ware, P. A.: This Journal, 92: 98, 1948.
4. Tolbert, B. M., Christenson, F., Chang, F. N-H., and Sah, P. P. T.: in press.
5. Finnegan, J. K., Haag, H. B., Larson, P. S., and Dreyfuss, M. L.: This Journal, 92: 269, 1948.
6. Dauben, W. G., Reid, J. C., and Yankwich, P. E.: Anal. Chem., 19: 828, 1947.
7. Reid, J. C., and Jones, H. B.: J. Biol. Chem., 174: 427, 1948.
8. Puharich, V., and Goetzl, F. R.: Permanente Foundation Med. Bull., 5: 19, 1947.
9. Friend, F. J., and Harris, S. C.: This Journal, 93: 161, 1948.
10. Gross, E. G., Holland, H., Carter, H. R., and Christensen, E. M.: Anesthesiology, 9: 459, 1948.
11. Eadie, G. S., Bernheim, F., and Fitzgerald, D. B.: This Journal, 94: 19, 1948.
12. Eisenbrandt, L. L., Abdou, I., Adler, T. K., and Elliott, H. W.: in preparation.
13. Scott, C. C., Chen, K. K., Kohlstaedt, K. G., Robbins, E. B., and Israel, F. W.: This Journal, 91: 147, 1947.
14. Scott, C. C., and Chen, K. K.: This Journal, 87: 63, 1946.

AN INVESTIGATION OF THE ACUTE TOXICITY OF THE OPTICAL ISOMERS OF ARTERENOL AND EPINEPHRINE

JAMES O. HOPPE, D. K. SEPPELIN, AND A. M. LANDS

Biology Division of the Sterling-Winthrop Research Institute, Rensselaer, New York

Received for publication January 4, 1949

The recent announcement of the successful resolution of arterenol (1, 2) raised a question with regard to the acute toxicity of its optical isomers in comparison with those of epinephrine. Most of the available information on the acute toxicity of epinephrine concerns the naturally occurring levo-isomer, although a few scattered reports on the dextro and racemic forms have appeared. In 1909, Cushny (3) reported the l-isomer of epinephrine to be 12–15 times as toxic as the d-form by subcutaneous injection into rats. Launoy and Menguy (4, 5) found the acute toxicity of l-epinephrine to be 20 times as great as the d-isomer when injected intravenously into rabbits. Marquardt and Koch (6), in a study of the acute subcutaneous toxicity of the optical isomers of epinephrine in mice, observed that l-epinephrine was 15 times as toxic as the d-isomer. Lands, Nash, Dertinger, Granger and McCarthy (7), also working with mice, showed that the acute toxicity of l-epinephrine was 1.7 times that of the racemic mixture by the intraperitoneal route.

Schultz (8) reported that demethylation of the amino group of epinephrine, to form the primary amine (arterenol) resulted in a decrease in toxicity. This investigator obtained acute subcutaneous toxicity values in mice of 12–16 mgm./kgm. and 40 mgm./kgm. for the racemic mixtures of epinephrine and arterenol, respectively. The present investigation was undertaken in an effort to determine the acute intravenous toxicity in mice and rats of levo, dextro and racemic arterenol in comparison with epinephrine.

METHODS. Aqueous solutions of the dextro and levo isomers and racemic mixtures of arterenol and epinephrine were used. The mortality data were calculated in terms of base. The solutions of l-epinephrine were prepared from U.S.P. Epinephrine Reference Standard powder according to the directions set forth in the U.S.P. XIII (9) and were used within an hour after preparation. The test animals were male albino mice of the Webster strain weighing 20 ± 2 gm. and Sherman strain male albino rats weighing 100 ± 10 gm. The volume of injection for each species was maintained at 0.35 ± 0.15 cc. and injected intravenously at a rate of 1.0 cc. per minute. Groups of 10 or more animals were injected at each dose level with dosages arranged at equal logarithmic intervals. Following injection, each group of mice was placed in a wire screen cage measuring 10 x 7 x 7 inches and the rats in similar cages with dimensions of 10 x 15½ x 7 inches in a room held at 24.5°C. Water and a nutritionally adequate diet were available at all times. Observations were made at frequent intervals but the LD_{50} ± its standard error calculated by the method of Miller and Tainter (10) was based on the total mortality both at 24 hours and 7 days.

RESULTS. The acute intravenous toxicity values obtained under the conditions described above are shown in table 1.

In rats, l-epinephrine is 20 times and in mice 18.5 times as toxic as d-epineph-

rine The acute toxicity of l-epinephrine is 1 8 times in rats and 1 5 times in mice that of the racemic mixture The acute toxicity ratio between l and d-arterenol is smaller than with epinephrine l-Arterenol is 14 times as toxic in rats and 12 times as toxic in mice as the d isomer No significant differences were observed between the toxicities of l- and d,l arterenol in either rats or mice

In rats intravenous injection of arterenol and epinephrine isomers produced mild to deep depression, blanching of the extremities, dyspnea, loss of muscular coordination terminating in a brief series of clonic convulsions and death following respiratory arrest Upon opening the thoracic cavity, the heart was ob-

TABLE I

Comparison of the acute intravenous toxicity of arterenol and epinephrine in rats and mice

COMPOUND	NUMBER OF ANIMALS USED	CONCENTRATION OF SOLUTION USED FOR INJECTION	$LD_{50} \pm$ S E EXPRESSED IN MGM /KGM OF BASE		TOXICITY RATIO AT 24 HOURS L-ISOMER = 1 0
			At 24 hrs	After 7 days	
Rats					
l Arterenol	150	0 0025–0 005	0 10 ± 0 01	0 10 ± 0 01	1 0
d Arterenol	80	0 06	1 40 ± 0 14	1 40 ± 0 14	14 0
dl Arterenol	120	0 005	0 13 ± 0 02	0 13 ± 0 02	1 3
l Epinephrine	75	0 001 –0 002	0 04 ± 0 004	0 04 ± 0 004	1 0
d Epinephrine	30	0 03	0 80 ± 0 08	0 80 ± 0 08	20 0
dl Epinephrine	45	0 002 –0 004	0 07 ± 0 003	0 07 ± 0 003	1 8
Mice					
l Arterenol	110	0 025 –0 10	5 ± 1	3 7 ± 0 6	1 0
d Arterenol	70	0 2 –0 8	60 ± 20	18 ± 5	12 0
dl Arterenol	120	0 025 –0 10	7 5 ± 2	4 7 ± 1	1 5
l Epinephrine	130	0 02	2 7 ± 0 2	2 5 ± 0 2	1 0
d Epinephrine	80	0 2 –0 4	50 ± 9	38 ± 9	18 5
dl Epinephrine	70	0 03 –0 06	4 0 ± 0 5	3 4 ± 0 3	1 5

served to beat for one to five minutes after cessation of respiration A bloody froth was invariably noted at the external nares immediately following the terminal convulsive seizure The lungs were turgid and filled with a frothy and usually bloody fluid The majority of deaths occurred within 10 to 20 minutes after injection with the l-isomers and within an hour after injecting the d-isomers or racemic mixtures With sublethal doses, the rats had fully recovered in 4 to 6 hours and there were no delayed deaths after 24 hours

The pattern of events in mice injected with the l isomers and racemic mixtures of arterenol and epinephrine was similar to that observed in rats However, with the d-isomers, death followed respiratory arrest which occurred over a period of several hours following injection A few mice died after 24 hours, resulting in a definite, but possibly statistically insignificant, decrease in the LD_{50} for the 7-day observation period The incidence of delayed deaths in mice was approximately

three times greater with arterenol than with epinephrine. The mice dying after the first 24 hours showed extensive hemorrhagic areas in the lungs, pulmonary edema, ascites, distention of the bladder with urine, which not infrequently contained flecks of bloody mucus, and paleness of the kidney cortex.

In rats the dose-mortality curves obtained with arterenol and epinephrine were very steep, as were also those in mice for both levo and racemic epinephrine. However, in mice the d-epinephrine and arterenol curves were very flat in comparison with those obtained with rats.

DISCUSSION. There were striking species differences between the sensitivities to these sympathomimetic amines as judged by lethal doses, since the rats were approximately 50 times as sensitive to arterenol and 60 times as sensitive to epinephrine as mice, and delayed deaths were produced only in mice. This might suggest that detoxication mechanisms for these amines are more efficient in the rat since recovery occurs promptly. However, since 50 to 60 times as much compound was injected per unit of body weight in mice their detoxication mechanisms were subjected to greater stress.

In rats the levo, dextro and racemic forms of epinephrine are 2.5, 1.8 and 1.9 times as toxic, respectively, as the same forms of arterenol. The mouse data show similar ratios, except that the d-isomer is difficult to evaluate because of the flatness of the dose-mortality curve. These results show that the inherent toxicity of epinephrine is approximately twice that of arterenol. This difference in the acute toxicity is smaller than is indicated by the data of Schultz (8), but agrees quite closely with the results obtained by Lands *et al.* (7) with the racemic mixtures of epinephrine and arterenol.

It is interesting to note that l-epinephrine is 1.8 times as toxic in rats and 1.5 times as toxic in mice as the racemic mixture. Both of these differences are statistically significant ($P = 0.01$). From a comparison of the median lethal doses, l-arterenol appears to be 1.3 times as toxic in rats and 1.5 times as toxic in mice as the racemic mixture; however, neither of these differences is statistically significant ($P = 0.18$ and $P = 0.27$, respectively). It would appear that the acute lethal effects of the racemic mixture of epinephrine result largely from the l-isomer. This does not appear to hold for racemic arterenol since the LD_{50} of l-arterenol is not significantly less than that of the racemic mixture, thereby indicating a significant contribution by the d-isomer to the acute lethal effects of the racemic mixture.

The very flat dose-mortality curves obtained in mice with arterenol and d-epinephrine, as indicated by the large standard errors, may be due to grouping of the mice following injection in view of the influence of aggregation on toxicity of sympathomimetic amines described by Chance (11, 12). Tainter *et al.* (1) observed that the toxicity of l-arterenol in mice is reduced when the animals are kept in individual cages, whereas segregation has no significant effect on the acute toxicity of l-epinephrine. This phenomenon has been ascribed to effects on the central nervous system, and suggests that perhaps the cause of death with arterenol may be more complex than with epinephrine. However, this requires more direct study for proper elucidation.

SUMMARY

1. Toxicity ratios for the levo, dextro and racemic forms of arterenol were found to be 1:14:1.3 in rats and 1:12:1.5 in mice by intravenous injection.

2. Toxicity ratios for the levo, dextro and racemic forms of epinephrine were found to be 1:20:1.8 in rats and 1:18.5:1.5 in mice by intravenous injection.

3. Rats were 50 times as sensitive to arterenol and 60 times as sensitive to epinephrine as mice when judged by the acute lethal doses.

4. The three optical forms of epinephrine were approximately twice as toxic as the corresponding forms of arterenol.

5. l-Epinephrine was significantly more toxic than the racemic mixture whereas no significant difference was found between l- and dl-arterenol in either rats or mice.

6. The dose-mortality curves were very steep in rats for arterenol and epinephrine and in mice for l, and d,l-epinephrine. However, they were flat in mice for d-epinephrine and arterenol.

REFERENCES

1. Tainter, M. L., Tullar, B. F., and Luduena, F. P.: Science, 107: 39, 1948.
2. Tullar, B. F.: J. Am. Chem. Soc., 70: 2067, 1948.
3. Cushny, A. R.: J. Physiol., 38: 259, 1909.
4. Launoy, L., and Menguy, B.: Compt. rend. Soc. de Biol., 83: 1510, 1920.
5. Launoy, L., and Menguy, B.: Compt. rend. Soc. de Biol., 87: 1066, 1922.
6. Marquardt, P., and Koch, R.: Arch. f. exper. Path. u. Pharmakol., 202: 658, 1943.
7. Lands, A. M., Nash, V. L., Dertinger, B. L., Granger, H. R., and McCarthy, H. M.: This Journal, 92: 369, 1948.
8. Shultz, W. H.: Hygienic Laboratory Bulletin, No. 55, April, 1909.
9. The Pharmacopoeia of the United States of America, Thirteenth Revision, 1947, Mack Printing Co., Easton, Pa., p. 198.
10. Miller, L. C., and Tainter, M. L.: Proc. Soc. Exper. Biol. and Med., 57: 261, 1944.
11. Chance, M. R. A.: This Journal, 87: 214, 1946.
12. Chance, M. R. A.: This Journal, 89: 289, 1947.

STUDIES ON THE FATE OF NICOTINE IN THE BODY

VI. Observations on the Relative Rate of Elimination of Nicotine by the Dog, Cat, Rabbit and Mouse

P. S. LARSON, J. K. FINNEGAN AND H. B. HAAG

Department of Pharmacology, Medical College of Virginia, Richmond 19

Received for publication January 7, 1949

In the course of our investigations on the fate of nicotine in the body, it became desirable to compare, as accurately as possible, the rate of elimination of nicotine by several species. The term "elimination" is used here in its broadest sense; that is, to imply any process whereby the drug is rendered physiologically inactive. In this sense, rate of elimination of nicotine by the rabbit has been studied by Weatherby (1), by the cat by Straub and Amann (2), and by the mouse by Heubner and Papierkowski (3). However, their techniques differ sufficiently in detail so that species comparison becomes difficult to make.

The results of our study follow.

Experimental. Two series of nicotine determinations were made on barbitalized animals; (a) LD_{50} values as determined by instantaneous intravenous injection of the total dose and (b) the lethal dose when given over an 8-hour period by continuous intravenous infusion.

The barbiturate used, dose and route of administration for each species studied were: dogs, dial 60 mgm. per kgm. intraperitoneally; cats, dial 60 mgm. per kgm. intraperitoneally; rabbits, sodium amytal 45 mgm. per kgm. intravenously; mice, dial 100 mgm. per kgm. intraperitoneally.

In the 8-hour experiments the nicotine concentration was adjusted with 0.9 per cent saline so that the dogs received 1 cc. of solution per minute, cats and rabbits received 0.2 cc. per minute, and mice received 0.01 cc. per minute.

Results. The intravenous LD_{50} values for nicotine under barbiturate anesthesia for the four species studied are shown in table I. Table II shows the lethal dose for the same species when given over an 8-hour period. Table III shows the calculated rate of elimination of nicotine by the four species under the conditions of the experiment.

Discussion. It is apparent from these results that a marked species variation exists in the ability of animals to eliminate nicotine. We wish to stress further that the results here given apply only to the experimental conditions used. They undoubtedly do not represent the maximum ability of these species to eliminate nicotine. In an earlier more detailed analysis for the dog (4), we showed that rate of elimination of nicotine (mgm. per kgm.) increased linearly with the dose for that fraction that is excreted unchanged in the urine and logarithmically with the dose for that fraction that is detoxified in the body. Since in the present experiments, elimination was dependent upon a continually increasing systemic concentration of nicotine over the 8-hour period, it is fair to assume that the maximum rate of elimination of nicotine by these species with-

out development of respiratory paralysis should be somewhat greater than the figures given in table III

TABLE I

Intravenous LD_{50} values for nicotine given in a single dose

DOG		CAT		RABBIT		MOUSE	
Dose	Mortality ratio	Dose	Mortality ratio	Dose	Mortality ratio	Dose	Mortality ratio
mgm/kgm		*mgm/kgm*		*mgm/kgm*		*mgm/kgm*	
3	0/4	1	0/2	8	0/6	6	3/10
4	1/6	1 5	2/6	9	1/6	7	4/10
5	3/6	2	3/6	10	5/6	8	7/10
6	4/4	2 5	6/6				
LD_{50} = 5 0 mgm/kgm		LD_{50} = 2 0 mgm/kgm		LD_{50} = 9 4 mgm/kgm		LD_{50} = 7 1 mgm/kgm	

TABLE II

Lethal doses for nicotine given intravenously over an 8 hour period

DOG		CAT		RABBIT		MOUSE	
Dose	Mortality ratio	Dose	Mortality ratio	Dose	Mortality ratio	Dose	Mortality ratio
mgm/kgm/8 hr		*mgm/kgm/8 hr*		*mgm/kgm/8 hr*		*mgm/kgm/8 hr*	
6	0/3	17	1/3	30	0/3	30	0/3
12	0/3	20	1/3	40	2/3	40	2/3
15	3/3	22	2/3	50	1/1	50	3/3
18	3/3	25	4/5			60	3/3
Lethal Dose = ca 15 mgm/kgm		Lethal Dose = ca 22 mgm/kgm		Lethal Dose = ca 40 mgm/kgm		Lethal Dose = ca 40 mgm/kgm	

TABLE III

Rate of elimination of nicotine

SPECIES	8 HOUR LETHAL DOSE	ACUTE LD_{50}	8 HOUR LETHAL DOSE MINUS ACUTE LD_{50}	NO OF LD_{50} DOSES ELIMINATED PER 8 HOURS
	mgm/kgm/8 hr	*mgm/kgm*	*mgm/kgm/8 hr*	
Dog	15	5 0	10	2
Cat	22	2 0	20	10
Rabbit	40	9 4	30 6	3 3
Mouse	40	7 1	32 9	4 6

It is of further interest to attempt to compare the relative abilities of these species to eliminate nicotine at comparable systemic concentrations of this material It is apparent that the cat which is killed by a single injection of 2 mgm per kgm. could not have reached as high a systemic concentration during the 8-

hour tests as did the rabbit which is killed by a single injection of 9.4 mgm. per kgm., and we have shown for the dog (4) that the absolute rate of elimination of nicotine increases as a function of its systemic concentration. In our judgment, the relative rates of elimination of nicotine by these species at equal systemic concentrations of the material can best be approximated by dividing the 8-hour eliminations for each by their respective LD_{50}'s for a single injection. This has been done in the last column of table III, and if valid indicates that, under equal conditions, the cat can eliminate nicotine 5 times as fast as the dog, the rabbit 1.7 times as fast, and the mouse 2.3 times as fast.

It is planned in the future to attempt to correlate these results with the capacities of the individual tissues of these species to detoxify nicotine (Warburg technique). If this is possible, similar studies on human tissue may permit of deductions concerning the capacity of man to eliminate nicotine.

SUMMARY

A comparison has been made of the rate of elimination of nicotine by the dog, cat, rabbit and mouse, and it has been shown that a considerable species variation exists in this regard.

REFERENCES

1. Weatherby, J. H.: Proc. Soc. Exper. Biol. & Med., 42: 593, 1939.
2. Straub, W., and Amann, A.: Arch. exper. Path. u. Pharmakol., 194: 429, 1940.
3. Heubner, W., and Papierkowski, J.: Arch. exper. Path. u. Pharmakol., 188: 605, 1938.
4. Finnegan, J. K., Larson, P. S., and Haag, H. B.: This Journal, 91: 357, 1947.

THE CONTROL OF EXPERIMENTAL PNEUMONIA WITH PENICILLIN

III. Inhalation Therapy of Established Pneumonia, as Related to Blood and Lung Levels

CATHERINE E. WILSON, STANLEY H. DURLACHER and ELEANOR A. BLISS

Medical Division, Army Chemical Center, Maryland and Johns Hopkins University Medical School, Baltimore, Maryland

Received for publication January 7, 1949

Some time ago we reported (1) that penicillin treatment of early lobar pneumonia in rats was as successful when the agent was administered as an inhalant as when it was given by injection. Later it was shown (2) that there was little difference between the blood levels achieved by the two methods of treatment. These studies have been extended to the therapy of established pneumonia in rats and to observations on the absorption and distribution of inhaled penicillin in normal and pneumonic animals.

Methods. *Animals.* White rats of the Wistar Institute strain, ranging in weight from 195 to 205 gm., were used.

Production of Pneumonia. Pneumonia was produced by the intrabronchial intubation of a mucin suspension of Type I pneumococci (Bailey strain) according to the modification of the technique of Nungester and Jourdonais (3) as described in the first paper. In this series the size of the infecting dose was increased to approximately 1000 pneumococci, instead of the 200 injected previously. Six hours after the injection, signs of pneumonia began to appear and by 24 hours there was consolidation of the entire cardiac lobe, or of 50 to 100 per cent of one of the other lobes, with septicaemia. By this time 4 per cent of the animals had died, the rest were evidently sick and 94 per cent of them, if not treated, succumbed in the next two or three days.

Penicillin. Crystalline sodium penicillin G was used in this study. We are indebted to Merck and Co., Inc. for giving us several million units of highly purified material, containing 1667 units per mgm. This was used for most of the blood level determinations and served as a standard of comparison for all other lots of penicillin. Solutions were made in $M/50$ phosphate buffer, pH 7.3.

Aerosolization. The mist was generated by a DeVilbiss 40 nebulizer at an airflow of 10 liters per minute. It was directed through the glass manifold used in the previous work and into a box, which was connected directly with the eight chambers of the manifold. The rats were placed singly in the sixteen compartments of a rectangular wire cage which just fitted in the box. Substitution of the box for the chambers of the manifold was made in order that more animals might be treated at one time, and with less difficulty. The mist in the box differed from that in the manifold. The concentration of penicillin was lower in the box and the particle size was smaller, having a mass median diameter of 0.5 μ.[1]

Assays. The concentration of the mist was determined by drawing a measured volume through a sintered glass-asbestos filter and analyzing the deposit for phosphate by the method of Fiske and SubbaRow (4). The efficiency of the filters was tested several times

---

[1] The authors are indebted to Miss G. Asset and Mr. J. Rosenbaum of the Aerosol Section, Medical Division, Army Chemical Center, for the determinations of particle size.

by drawing the mist through two filters, arranged in series. Very little phosphate was found on the second filter—only 2 to 3 per cent of the amount found on the first—indicating that even these very small particles were retained by this type of filter. Evaporation caused the concentration of penicillin and phosphate in the nebulizer and consequently in the mist, to rise by as much as 30 per cent during a 20-minute run. For this reason two analyses of the mist were usually made during each run and the average was taken as representing the concentration.

Penicillin levels were determined by the broth dilution method, using the C203 strain of haemolytic streptococcus, as previously described (2).

In order to determine the penicillin content of the lower respiratory tract the rats were killed with ether, the trachea was tied off at the larynx and the trachea, bronchi and lungs were removed en masse. If the trachea or pneumonic lobe was to be treated separately, the major bronchi were individually ligated and severed below the carina. The tissue was debrided, weighed, and minced in a Waring blendor for 2 minutes, in 20 or 50 cc. of saline solution. The creamy emulsion was filtered through a Seitz filter and the filtrate was titrated for penicillin. Recovery of penicillin by this technique was controlled by adding known amounts of the agent to normal and consolidated lung tissues and processing them in the same way. All of the added penicillin was accounted for in the filtrate.

TABLE I

Penicillin treatment of pneumonia of 24-hours duration in rats

METHOD OF ADMINISTRATION	DOSE*	NUMBER OF RATS	NUMBER THAT DIED	MORTALITY
				per cent
Inhalation of aerosol..........	20 min. exposure, 400 u/liter	63	13	21
Intramuscular injection.........	500 u	44	18	41
Intramuscular injection.........	250 u	35	17	49
Untreated controls............	—	46	43	93.5

* Per treatment; 4 treatments given.

Schedule of Therapy. In the therapeutic comparisons the rats received 4 treatments—at 24, 30, 36 and 48 hours after they had been infected with the pneumococci. Each inhalation treatment consisted of a 20-minute exposure to a mist containing approximately 400 units of penicillin per liter of air. To obtain this mist concentration the solution in the nebulizer had to contain 60,000 units per cc. The intramuscular injections consisted of either 500 or 250 units administered 4 times.

RESULTS. *Therapy.* In these rats, in which pneumonia had been allowed to progress to the point where there was extensive consolidation of lung tissue, therapy with penicillin was successful in saving at least 50 per cent. As shown in table I, four 20-minute exposures to aerosols containing 400 units of penicillin per liter of air reduced the mortality to 21 per cent. With 4 intramuscular injections of 250 and 500 units each the mortality was 49 and 41 per cent respectively. The difference between the 21 and the 41 per cent is statistically significant, having a 3 per cent probability of chance, and one can therefore conclude that the aerosol therapy was more effective than the injection therapy in the doses used here.

Blood Levels. In table II are shown the levels of penicillin noted in the blood of normal rats, and rats with pneumonic consolidation, at intervals up to 2 hours

after they had been given a single treatment either by intramuscular injection or by inhalation. The intervals are dated from the time when treatment was completed. The injection of 500 units resulted in higher blood levels than occurred after a 20-minute exposure to a 400 unit per liter aerosol.[2] This was true of both healthy and diseased rats. The difference between the effects of the two modes of administration was particularly marked in the initial blood level and in that

TABLE II

Blood levels in normal and pneumonic rats after single treatment with penicillin G

TIME AFTER END OF TREATMENT	MODE OF ADMINISTRATION AND DOSE					
	20 Min. inhalation of 400 u/liter—Aerosol		Intramuscular injection			
			500 units		250 units	
	Normal	Pneumonic	Normal	Pneumonic	Normal	Pneumonic
minutes		*Unit per cc. of whole blood**				
5	.575	.195	1.68	1.41	—	—
15	—	—	.794	.904	.330	.397
30	.199	.195	.426	.617	.144	.250
60	.125	.092	.147	.210	.057	.067
120	.024	.038	.015	.021	<.015	<.015

* Geometric means; 5 to 14 rats for each point.

TABLE III

*Penicillin within the lower respiratory tract following aerosolization**

STATUS OF RATS	TIME AFTER Rx	NO. OF RATS	UNITS RECOVERED									WEIGHT OF TISSUE IN GM. (MEAN)	UNITS PER GM. OF TISSUE (MEAN)
			100	50	25	20	10	5	2.5	1.25	Geom. mean		
			Number of rats										
	min.												
Normal.....	5	8	6	2							84.1	1.288	65.3
Pneumonic..	5	8		2	5		1				26.5	2.396	11.1
Normal.....	60	6				1	3	1	1		7.9	1.274	6.2
Pneumonic..	60	6					1	2	1	2	3.2	2.176	1.4

* 20-minute exposure to 400 u/liter mist.

determined 30 minutes later. Immediately after treatment by inhalation there was a marked difference between the blood levels in the normal and pneumonic animals. In the animals which received penicillin by injection this difference did not exist, although the drug appeared to leave the blood more slowly in the pneumonic rats. Penicillin also disappeared from the blood more slowly following inhalation than following injection.

Lung Levels. In the preliminary tests to determine the relative amounts of penicillin which reached the lower respiratory tract of normal and pneumonic

[2] Differences between blood levels observed here and those reported previously are explained by differences in the type of penicillin and the character of the aerosol.

rats after inhalation, the lungs, bronchi and trachea were treated as a unit. From the data in table III one sees that approximately 3 times as much penicillin was recovered from the lower respiratory tract of normal animals as was recovered from the tissue of pneumonic rats. In both instances, however, high concentrations of penicillin were found and although these declined sharply with time, they were still well above the so-called "therapeutic level" at the end of one hour. Because of the greater density of consolidated tissue the difference between the levels in normal and pneumonic rats is enhanced when the data are expressed in terms of units per gm. of lung tissue.

TABLE IV

*Distribution of penicillin in the lower respiratory tract 5 minutes after aerosolization**

STATUS OF RATS	EXTENT OF CONSOLIDATION	PENICILLIN RECOVERED FROM							
		Trachea		Consolidated Lobe		Remainder		Total	
		Units	u/gm.†	Units	u/gm.	Units	u/gm.	Units	u/gm.
Pneumonic	Rt. lower, 50%	10	22.9			40	21.2	50	21.5
Pneumonic	Cardiac, 100%	5	19.6	0.3	0.3	20	20.9	25.3	11.7
Pneumonic	Left, 40%			5.0	6.4	25	29.6	30	18.4
Pneumonic	Rt. lower, 50%			1.2	1.5	20	17.5	21.8	9.8
Pneumonic	Rt. middle & Cardiac, 100%	5	14.2	0.6	0.85	10	9.5	15.6	7.4
Pneumonic	Left, 90%	5	16.9			20	11.7	25.0	12.4
Pneumonic	Left, 80%	10	31.2	2.5	1.8	20	23.5	32.5	12.7
Normal		2.5	9.6			160	138.5	162.5	114.8
Normal		2.5	12.2			50	50.5	52.5	43.9
Normal		5	13.3			100	78.7	105	63.8

* 20-minute exposure to 400 u/liter mist.

† Units per gm. of tissue.

It was desirable to determine how inhaled penicillin was distributed in the lower respiratory tract with special attention to deposition at the bifurcation of the trachea and to relative penetration of healthy and diseased tissue. In order to do this the tracheas, the lobes showing consolidation and the healthy tissues were processed separately. The findings are shown in table IV. In normal rats the amount of penicillin deposited in the trachea was only a small fraction of the total recovered from the lower respiratory tract. Not only was the absolute recovery from the trachea higher in pneumonic than in normal rats, but it represented a greater proportion of the total recovery from the lungs.

In pneumonic rats the amount of penicillin found in the consolidated lobe was considerably less than that found in the aerated lobes. Where partial consolidation existed slightly higher values were obtained. The aerated lobes of the lungs of pneumonic rats had high concentrations of penicillin but not as high as those found in the normal rats.

When 500 units were injected intramuscularly into 2 normal and 2 pneumonic rats, 1 pneumonic rat had, at 25 minutes, a lung level of 1.2 unit per cc. No penicillin was detectable in the lungs of the other 3 rats. In order to obtain lung levels comparable with those observed after inhalation, 25,000 units had to be injected. With these high doses the pneumonic lungs contained more penicillin than the normal, as might be expected since there was more blood and exudate in the consolidated areas.

Discussion. Twenty-four-hour pneumonia in rats was more successfully treated by inhalations of penicillin than by injections. The aerosolization treatment employed here was calculated to result in a retention, in normal rats, of 400–500 units of penicillin, or an amount roughly equivalent to the dosage by injection. To ascertain whether differences in drug distribution would explain the differences in the therapeutic results obtained by the two routes, concentrations of penicillin in the blood and respiratory tract were determined.

Blood levels in both aerosolized and injected groups were within the so-called therapeutic range but the highest values were observed in the group given intramuscular injection. Rats receiving penicillin by aerosol had lower initial blood levels but a larger percentage of the initial level was maintained two hours after treatment. Furthermore, during the period of aerosolization the rats were receiving penicillin for an additional 20 minutes. Nevertheless, the better therapeutic results following inhalation could not have been due solely to the amount of penicillin reaching the blood, for higher blood levels were found in the group with the greater mortality.

In our previous work, retained dose following aerosolization was estimated from data obtained in normal animals. The respiratory pattern of rats with pneumonia of 6-hours duration showed no significant deviations from those of the normal controls, and the retained dose calculated for the normal animal was considered the therapeutic dose. The breathing pattern of rats with 24-hour pneumonia differed markedly from that of normal rats; respirations of the former were extremely shallow and rapid. This type of breathing is known to diminish retention. To gain some idea of how much penicillin was retained by pneumonic rats following aerosolization, blood and lung levels of such animals and normals were compared. The blood and lung levels found in pneumonic rats were about one-third as high as those in normals. It is reasonable to conclude that the retained dose was also about one-third as high in pneumonic as in normal rats. With this in mind, the better therapeutic effect of the aerosol was all the more striking.

The explanation of the more efficient therapy obtained by aerosolization may be found in the distribution of penicillin in the respiratory tract. The pneumonic rats had 25 times as much penicillin in their lungs and trachea after inhalation as after intramuscular injection. In the normal rats 50 to 100 times more penicillin was found in the respiratory tract after inhalation than after injection of a comparable dose. In both normal and pneumonic rats, one hour after inhalation, appreciable amounts of penicillin persisted in the lung. This observation was in agreement with the findings of Laurent *et al.* (5) in normal

rats, although these workers reported higher calculated retained doses and lower blood and lung levels than were noted here.

In pneumonic rats following inhalation only a small fraction of the total penicillin in the lower respiratory tract could be detected in the consolidated lobe of the lung; approximately $\frac{1}{3}$ to $\frac{1}{5}$ of the total was in the trachea; the major portion was in the unconsolidated lobes. The concentration of penicillin in the aerated portions of the lung was high when considered on the basis of units of penicillin per gm. of tissue. This reservoir of penicillin probably acted to maintain the lood levels and to prevent extension of the pneumonia into uninvolved portions of the lung through the respiratory passages.

After intramuscular injection of 500 units very small quantities of penicillin were recovered from the lungs of normal or pneumonic rats. The fact that over 50 per cent of the rats receiving such therapy survived indicates that cures can be effected without high lung concentrations. The significantly lower mortality rate following aerosolization suggests that high lung penicillin levels are of benefit in the therapy of pneumococcal pneumonia in rats.

SUMMARY

In pneumococcal pneumonia of rats, untreated for 24 hours, 4 intramuscular injections of 250 and 500 units of penicillin reduced mortality from 94 per cent observed in controls, to 49 and 41 per cent, respectively. With 4 inhalations of a mist containing 400 units of penicillin per liter of air the mortality was 21 per cent. The difference between the mortality rates after aerosolization and after intramuscular injection is statistically significant.

Initial blood levels of penicillin in pneumonic rats were higher following intramuscular injection than after exposure to aerosol, but levels were better maintained in aerosol treated animals. Normal rats exposed to the same penicillin aerosol had blood levels 3 times as high as rats with pneumonia of 24 hours duration.

Penicillin levels in the respiratory tract of pneumonic rats following aerosolization were more than 25 times as high as levels in rats treated by intramuscular injection. Normal rats exposed to the same penicillin aerosol had lung levels 3 times as high as pneumonic rats. After exposure of rats to this particular aerosol only a small fraction of the penicillin retained was in the trachea; the largest portion was in the structures distal to the carina. In pneumonic rats exposed to aerosol there was only a trace of penicillin in the consolidated portions of the lung.

ACKNOWLEDGMENT. The technical assistance of Mr. Joseph S. Wiles is gratefully acknowledged.

REFERENCES

1. WILSON, C. E., HAMMOND, C. W., BYRNE, A. F., AND BLISS, E. A.: Bull. Johns Hopkins Hospital, 77: 411, 1945.
2. WILSON, C. E., HAMMOND, C. W., BYRNE, A. F., AND BLISS, E. A.: Bull. Johns Hopkins Hospital, 79: 451, 1946.
3. NUNGESTER, W. J., AND JOURDONAIS, L. F.: J. Infect. Dis., 59: 258, 1936.
4. FISKE, C. H., AND SUBBAROW, Y.: J. Biol. Chem., 66: 375, 1925.
5. LAURENT, A. M., MCILROY, A. P., AND HADLEY, F. P.: Proc. Soc. Exper. Biol. and Med 68: 213, 1948.

THE RELATIONSHIP BETWEEN CHOLINESTERASE INHIBITION AND FUNCTION IN A NEURO-EFFECTOR SYSTEM[1]

WALTER F. RIKER AND W. CLARKE WESCOE

Department of Pharmacology, Cornell University Medical College, New York City

Received for publication January 7, 1949

The role of cholinesterase (ChE) in the maintenance of vital processes has been the subject of a number of recent investigations. These investigations were given impetus by the advent of di-isopropyl fluorophosphate (DFP), a potent and irreversible inactivator of ChE, which made possible, for the first time, absolute determinations of degrees of ChE inactivation (1).

Mazur and Bodansky (1) were the first to make the point that, in rabbits and monkeys exposed to lethal amounts of DFP, death was associated with levels of brain ChE approaching zero. In a subsequent study Koelle and Gilman (2) reported that for rats to survive and appear normal it was necessary for 10 to 20 per cent of the average normal brain ChE activity to be retained. These results have been confirmed recently by Nachmansohn and Feld (3) who stated that death of animals always occurs when brain ChE activity approaches zero; conversely, in the brains of surviving animals ChE is always present, although it may be in a concentration as low as 10 per cent of the average normal activity. These latter investigators pointed out, however, that such experiments on the whole animal are not favorable for the determination of the minimum ChE activity necessary to maintain normal function because so many centers of different sensitivity are involved. They referred further to unpublished experiments on frog sciatic nerve *in vitro*, in which it was possible to abolish 92 per cent of the initial ChE activity before conduction was impaired. In addition, the results of Bullock *et al.* (4) indicated that conduction in isolated squid and lobster nerve requires the presence of ChE; they found that 80 per cent of the ChE could be removed before the action potential was affected. These findings indicated that ChE is present in abundance in neural structures, and that an extremely low concentration of the enzyme may be sufficient to maintain function. On the other hand, it must be pointed out that investigations *in vitro* are likewise subject to criticism, in that it is not always possible to carry over these results to conditions *in vivo*.

The present report is concerned with experiments designed to determine the relationship between ChE activity and function in a simple neuro-effector system of the intact animal.

The chemical nature of transmission at parasympathetic junctions has been dem-

[1] The work described in this paper was done in part under contract between the Medical Division, Chemical Corps, U. S. Army, and Cornell University Medical College. Under terms of the contract, the Chemical Corps neither restricts nor is responsible for the opinions or conclusions of the authors.

onstrated adequately and has been defined particularly well for the salivary gland (5–10). Certain of these studies indicated that a cholinergic substance is liberated in considerable quantity during chorda tympani stimulation in the presence of physostigmine (5, 7–10). Therefore, the chorda tympani-submaxillary gland system provides a well-established basis for the study of the quantitative relationships between cholinergic function and ChE activity *in vivo*. It provides, therefore, a suitable technique for the evaluation of anti-ChE compounds.

METHODS. All experiments were performed on cats previously anesthetized with intraperitoneal Dial-Urethane solution (0.6 cc./kgm.). From each experimental animal the left submaxillary gland was removed for the determination of control ChE activity. Subsequently each animal was prepared as follows: The trachea was cannulated and the right common carotid artery exposed for injection. The right chorda tympani nerve was isolated and severed from its central connections, and, in the experiments in which electrical stimulation was performed, silver wire electrodes were applied to the distal portion. The right main submaxillary duct was cannulated with a blunt 22-gauge needle and connected to a drop recorder. The recorder served to activate a signal magnet which recorded on a smoked drum. The DFP solution was prepared freshly in saline and each dose was adjusted to a volume of 0.1 cc./kgm. All injections were made into the right carotid artery with a 26-gauge needle. On the completion of an experiment the right submaxillary gland was removed for the determination of the final ChE activity.

Electrical stimulation of the chorda tympani nerve was accomplished with an Electrodyne stimulator.[2] The output of this stimulator has a constant wave form independent of the intensity and frequency of the stimulus. The voltage employed was maintained at a supra-maximal intensity of approximately 200 volts. The frequency of the stimulus varied with the individual experiment and ranged from single pulses to 30 pulses per second.

The ChE activity of the glands was determined manometrically with the Warburg apparatus. Each gland was washed thoroughly with saline, freed of extraneous tissue and weighed while moist. The entire gland was homogenized in 0.03 *M* $NaHCO_3$ in a Waring blendor; the final homogenate dilution was 1:60. To the main compartment of the Warburg vessel was added 3.0 cc. of the gland homogenate and 0.5 cc. of distilled water; to the side bulb was added 0.5 cc. of 0.12 *M* solution of acetylcholine bromide in 0.03 *M* $NaHCO_3$. The vessels were equilibrated with a mixture of 95 per cent N_2 and 5 per cent CO_2 and allowed to come to thermal equilibrium in the bath at 38°C. The reaction was begun by tipping and readings were made at 10 minute intervals for 60 minutes. The results were calculated as the cmm. CO_2/30 min./50 mgm. wet weight of tissue.

RESULTS. *The Effect of Intra-arterial DFP on the ChE Activity of the Submaxillary Gland.* The effect of serial intra-arterial doses of DFP on the ChE activity of the submaxillary gland was studied on 44 cats. The results are shown in figure 1A. It can be seen from the curve that an initial dose of 20 microgm./kgm.[3] reduced the ChE activity of the gland to 51 per cent of the control value, whereas subsequent increments of 25 or 50 microgm./kgm. produced a lesser degree of esterase inhibition. For example, a dose of 150 microgm./kgm. which reduced the ChE activity to 6 per cent of the control level, when repeated produced but a slight further depression of the activity. It is apparent that with additional large increments of dose the ChE activity gradually approaches the limiting value of zero.

[2] Manufactured by the Electrodyne Company, Boston, Mass.

[3] All doses in this report were calculated on a body weight basis.

From these data it can be said that the effect of a given dose of DFP on the ChE activity of the gland is proportional to the level of esterase activity existing at the time of the dose. Assuming that the inactivation of ChE by DFP is ir-

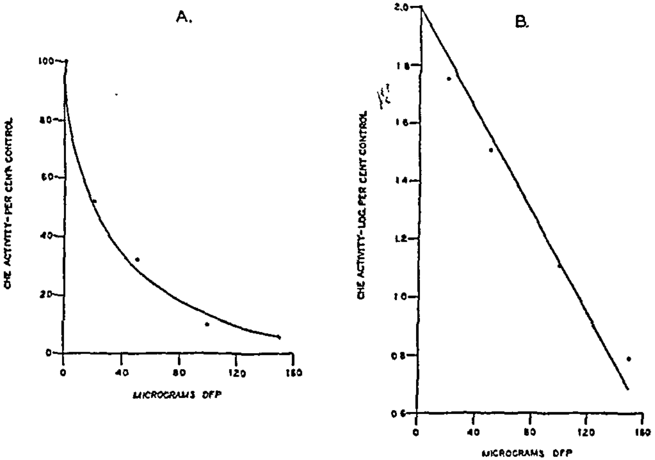


FIG. 1. THE EFFECT OF INTRA-CAROTID DFP ON THE SUBMAXILLARY GLAND CHE

reversible, the relationship between dose and enzyme activity *in vivo* may be expressed as:

$$-\frac{dE}{dx} = k_1 E \qquad \text{(I)}$$

where E represents the ChE activity and x the DFP dose. Upon integration equation (I) becomes:

$$k_1 = \frac{2.303}{x} \log \frac{E_0}{E_t} \qquad \text{(II)}$$

where E_0 is the ChE activity at the time of a given dose of DFP and E_t the activity after the dose.

When the logarithm of the ChE activity of the gland is plotted against the dose of DFP (between the dose limits of 0 and 150 microgm./kgm.), a linear relationship is observed (figure 1B). The line fitted by the method of least squares gives a value for $k_1 = -0.0086$. The value, k_1, gives the average rate of inactivation of the salivary gland ChE per microgm./kgm. of DFP injected.

The Relationship Between ChE Activity and the Threshold of the Gland to Electrical Stimulation. This relationship was studied in 6 cats. In each experiment

a recording was made of the salivary flow in response to stimulation of the chorda tympani for a period of 30 seconds; the stimuli were of supra-maximal intensity but of varying frequency. The minimal frequency of stimulation to which the gland would respond was determined for each cat. Subsequent to this a dose of 20 microgm./kgm. of DFP was administered intra-arterially and the threshold re-determined. This procedure was repeated after each additional dose of 20 microgm./kgm. until a total dose of 100 microgm./kgm. had been given.

The results are shown in figure 2A. The figure shows that up to a dose of more than 20 microgm./kgm. of DFP there was no change in threshold; a dose of 100 microgm./kgm. produced a maximal reduction of the threshold. It is evident (see figure 1A) that at least 50 per cent of the initial ChE activity of the gland must be inhibited before the threshold to nerve stimulation is affected, and that

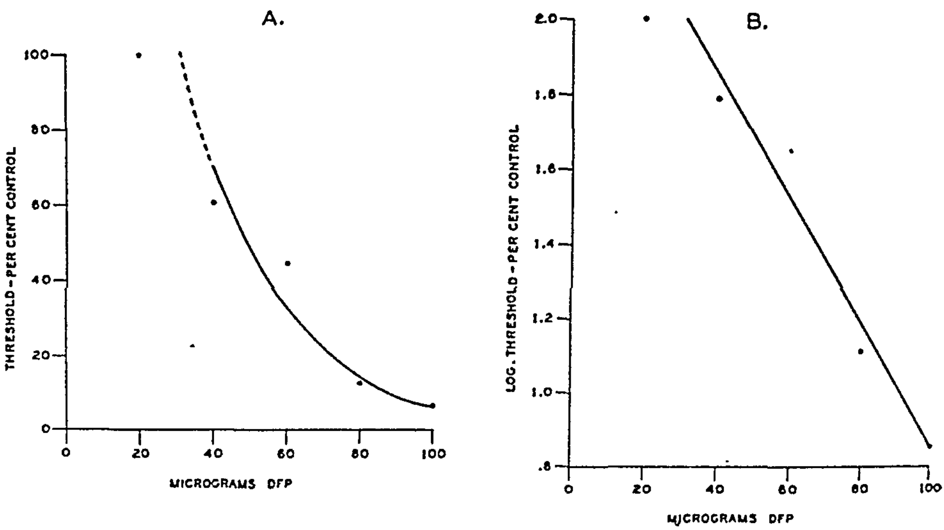


FIG. 2. THE EFFECT OF INTRA-CAROTID DFP ON THE THRESHOLD OF THE SECRETORY RESPONSE TO CHORDA TYMPANI STIMULATION

The frequency of maximal pulses required to elicit a response in the untreated cat is taken as 100 per cent.

the maximal change in threshold is reached when the ChE activity is 10 per cent of normal.

When the logarithm of the threshold frequency (expressed as per cent of control value) is plotted against dose of DFP a linear trend is evident. This plot is shown in figure 2B. The relationship between dose of DFP and threshold to electrical stimulation of the salivary gland is similar to that between dose of DFP and ChE activity. This relationship can also be formulated:

$$-\frac{dT}{dx} = k_2 T \qquad \text{(III)}$$

where T represents threshold value and x is the DFP dose. The regression line in figure 2B, fitted by the method of least squares, gives a value for $k_2 = -0.017$.

The relationship between the ChE activity of the gland and its threshold to electrical stimulation can be formulated:

$$-\log T = K(-\log E) \qquad (IV)$$

where K is equal to $\frac{k_1}{k_2}$ or 0.51. This relationship is shown in figure 3. The relationship implies that the minimal effective acetylcholine concentration at the

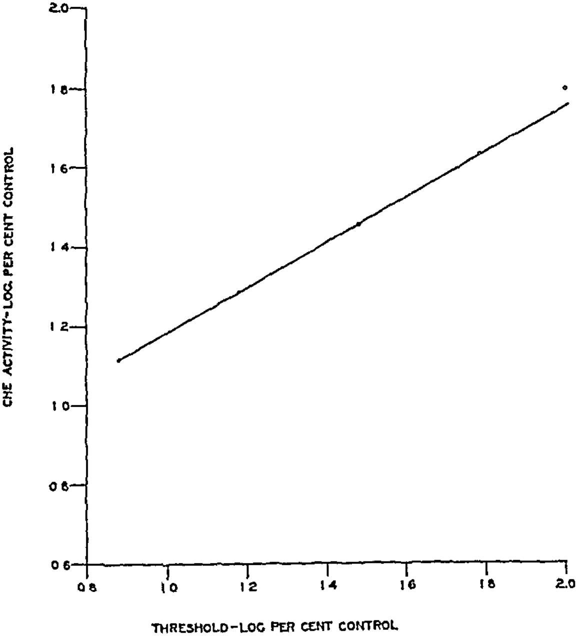


FIG 3. THE RELATIONSHIP BETWEEN THE ChE ACTIVITY OF THE SUBMAXILLARY GLAND AND ITS THRESHOLD TO NERVE STIMULATION

junction is a function of the ratio of stimulus frequency to ChE activity. This is in accord with the conclusions of Beznak and Farkas (11), that the production of acetylcholine at this neuro-effector junction is a function of the frequency of chorda stimulation.

The Effect of DFP on the Response of the Salivary Gland to Electrical Stimulation. This effect was studied in 5 cats. The chorda tympani was stimulated for a period of 30 seconds with shocks of supra-maximal intensity and at a frequency

which produced a maximal response. The total response of the gland was recorded; the total response is defined as the secretion that occurs during and after stimulation.

In the untreated cat, secretion subsided abruptly with the cessation of the stimulus. This observation is in accord with the course of salivary secretion in response to electrical stimulation as defined quantitatively by Maltesos and Weigmann (12).

An initial intra-arterial dose of 20 microgm./kgm. of DFP did not alter the response of the gland. However, at this point an additional dose of 20 microgm./kgm. produced an increase of the total response of the gland to the fixed stimulus. The total response reached a maximum after a dose of 100 microgm./kgm. had been given in increments of 20 microgm./kgm. The range of ChE activity in which potentiation of the total response occurred corresponds to the limits of ChE activity (50 → 10 per cent) in which the minimal and maximal changes in the threshold occurred. However, the increase in the total response at the point of the maximal DFP effect was confined predominantly to the post-stimulatory phase of secretion which increased 455 per cent in contrast to 21 per cent in the response during stimulation. The course of secretion during actual stimulation was altered but slightly despite profound depression of the ChE activity. This supports the findings of Maltesos and Weigmann (12) who failed to observe an effect of physostigmine on the course of secretion during stimulation.

When the logarithm of the per cent increase in the response is plotted against the dose of DFP a curve results which has the form of a rectangular hyperbola (figure 4); in this curve the response approaches a limiting value asymptotically. This hyperbolic relationship is analogous to the formulations (summarized by Clark, 13) for the responses of several different effector systems to varying acetylcholine concentrations. Therefore, the data of figure 4 suggest that the progressive decrease in the glandular ChE activity is associated with a prolonged action of the effective acetylcholine concentration at the neuro-effector junction.

Beznak and Farkas (11) found that the response of the salivary gland to different intra-arterial doses of acetylcholine produced a curve having the form of a rectangular hyperbola. Eserine merely decreased the threshold of the gland to acetylcholine, and the same asymptotic deflection was approached in both cases. In accord with these findings, a similar series of experiments was performed on 4 cats to compare the response of the gland to a fixed intra-arterial dose of acetylcholine at various levels of ChE activity. The results are shown in figure 5; the logarithm of the per cent increase in response to the acetylcholine injection is plotted for each DFP dose. No attempt was made to adjust the acetylcholine response to that resulting from electrical stimulation, so that the curves illustrated in figures 4 and 5 differ from each other quantitatively. It is evident, however, that the form of the response to acetylcholine is the same as that observed with the electrical stimulation. It is concluded, therefore, that the curves of figures 4 and 5 indicate a greater effective acetylcholine concentration acting over a prolonged period.

In previous studies concerned with the response of various effector systems to

acetylcholine it has become evident that as the acetylcholine concentration increases, the response approaches a maximum asymptotically. This appears to be the case in the response of the gland to either electrical or chemical stimulation at progressively lower levels of ChE activity. However, this is true only to a limit of 100 microgm./kgm. of DFP, or in terms of ChE activity, when it is reduced to 10 per cent of control value. At this point the administration of more DFP results in a sharp increase in the response to electrical stimulation and in the response to the fixed dose of acetylcholine. Therefore, what appears to be an asymptotic deflection in the curves of figures 4 and 5 is in reality a point of

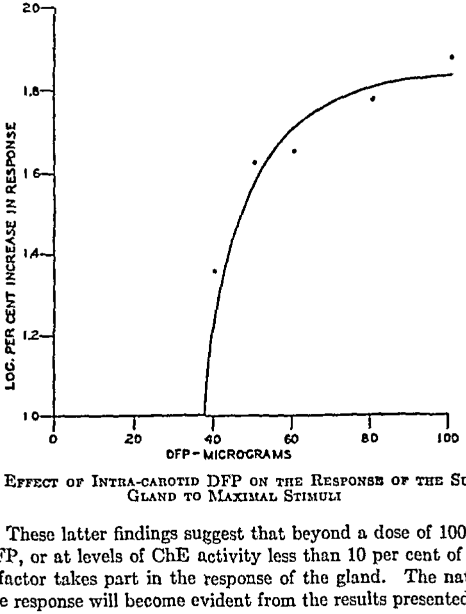


FIG. 4. THE EFFECT OF INTRA-CAROTID DFP ON THE RESPONSE OF THE SUBMAXILLARY GLAND TO MAXIMAL STIMULI

inflection. These latter findings suggest that beyond a dose of 100 microgm./kgm. of DFP, or at levels of ChE activity less than 10 per cent of normal, an additional factor takes part in the response of the gland. The nature of this phase of the response will become evident from the results presented in the following section.

The Production of Spontaneous Salivary Activity by DFP. The ability of DFP to produce spontaneous salivary secretion was studied on 17 cats. Of these cats, 6 were given single intra-arterial injections of DFP and their salivary response recorded. The remainder were given repeated injections of 100 microgm./kgm. of DFP at 30-minute intervals and the response recorded; the injections were usually continued until a maximal rate of flow was attained. It is noteworthy that spontaneous salivary secretion was never observed in the untreated cat.

Although the response to each dose of DFP varied widely in different animals, the initial dose of 100 microgm./kgm. proved to be, in the average, an approximately threshold dose for instituting salivary flow, and similarly a dose of 800 microgm./kgm. was, in the average, the dose at which the maximal rate of salivary secretion was obtained. Similar results were obtained in the dog by Brassfield *et al.* (14) with the repeated intra-arterial injection of DFP. The maximal rate of secretion approached but never exceeded the maximal response to stimulation.

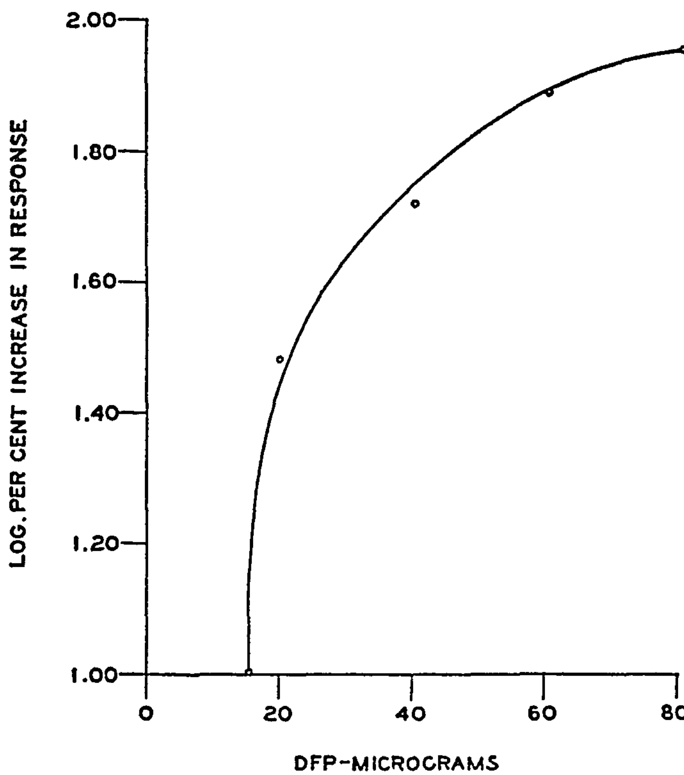


FIG. 5. THE EFFECT OF INTRA-CAROTID DFP ON THE RESPONSE OF THE SUBMAXILLARY GLAND TO A FIXED INTRA-CAROTID DOSE OF ACETYLCHOLINE

When the average rate of salivary flow (expressed as drops/hour) for each dose increment is plotted against the logarithm of the DFP dose, a linear relationship is obtained (figure 6). The figure shows that spontaneous activity of the gland does not occur until a dose of 100 microgm./kgm. of DFP is given. This dose corresponds to an amount sufficient to reduce its esterase activity to approximately 10 per cent of the control. The fact that additional doses of DFP continue to increase the rate of salivary flow indicates that continuing destruction of the remaining 10 per cent of the ChE activity of the gland occurs and that, in accord with the data presented in figure 1, relatively large dose increments are now necessary to effect a virtual abolition of the ChE activity.

Although the exact quantitative nature of the relationship between dose increment and ChE decrement is not ascertainable in this range, each percentile decrease in ChE activity from 10 per cent toward zero exerts a pronounced effect on the stability of this neuro-effector system. It is noteworthy that a refractory state of this parasympathetic structure was not observed despite maximal inactivation of ChE by DFP. This is in contrast to the effects of DFP on neuro-muscular (15, 16) and ganglionic (17) transmission.

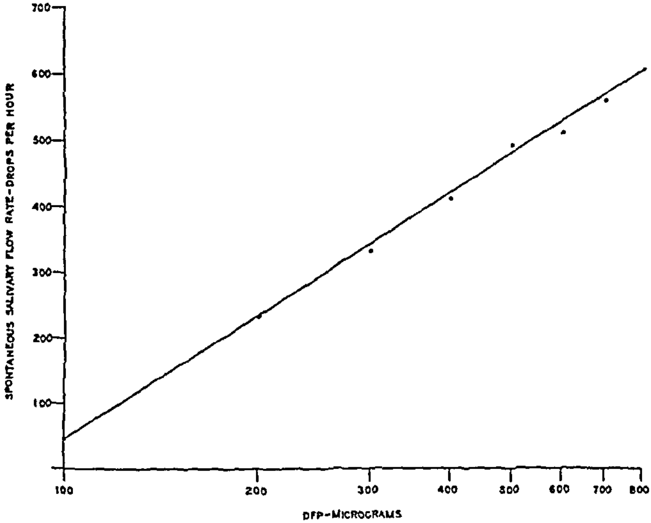


FIG. 6. THE EFFECT OF INTRA-CAROTID DFP ON THE RATE OF SPONTANEOUS SALIVARY SECRETION BY THE SUBMAXILLARY GLAND

With these data in mind it is relevant to re-examine certain of the data of the preceding sections. In this regard it is to be noted that a maximal decrease of approximately 93 per cent in the threshold of the gland to nerve stimulation occurred when the ChE activity was reduced to 10 per cent of the control value and that it was at this precise point where spontaneous activity began. At this point it was not possible to evaluate further changes in threshold. It is to be noted also that this point of 10 per cent ChE activity is the value at which the potentiation of the response to maximal stimulation approached its asymptotic deflection. It was pointed out (*vide supra*) that the continued reduction of ChE activity from this level caused a sharp positive inflection in the curve of potentiation. It is evident, then, that this phase of the response to either electrical or chemical stimulation represents a summation of effects, and, therefore, the

point of maximal potentiation of nervous stimuli is reached immediately prior to the onset of spontaneous activity.

Discussion. The results presented in this report constitute the first demonstration *in vivo* of the function of a neuro-effector system at various levels of its ChE activity. It has been reported that in man and laboratory animals it is possible to inactivate the serum ChE completely with no evident deviation from the normal state (1, 2, 18, 19); it is evident, therefore, that the serum ChE activity is unable to serve as a guide to the pharmacological effects of ChE inhibitors. The attempt to use erythrocyte ChE activity as an indicator for the action of anti-ChE compounds has not provided any definite correlation between the degree of enzyme inhibition and alteration of function (18, 19). The only definite correlation which has been established has been between lethal outcome and brain ChE activity (1, 2, 3); in this case the critical level appears to be approximately 10 per cent of the original level. The data of the present investigation indicate that a complete breakdown in the stability of the submaxillary gland ensues when the level of ChE activity reaches or falls below 10 per cent of normal. This represents the final drastic effect of maximal inactivation of junctional ChE and is directly comparable to the fatal effect of reducing the brain ChE to 10 per cent or less.

It would be reasonable to assume that a progressive decrease in tissue ChE would be accompanied by progressive changes in physiological function; this assumption, however, has been shown by the present studies to be only partially true. The data presented in figures 2, 4, and 5, show that the initial reduction in ChE activity from 100 to 50 per cent is not accompanied by any change in salivary function; from this point, however, further reduction in ChE activity is associated with progressive changes in function. These functional alterations which occur at the initial critical level of ChE inhibition are a progressive lowering of the effector's threshold to electrical stimulation and a progressive increase in the magnitude of its response to electrical and chemical stimulation. There is, then, an initial level of ChE activity which is critical (50–35 per cent of normal) for the onset of functional alteration in response to stimulation. The fact that more than one-half of the original ChE activity can be removed without affecting function suggests that this fraction is either physiologically dispensable or not concerned with transmission.

The threshold of the response to nerve stimulation must depend on the delivery of a minimal effective concentration of acetylcholine to the effector cell. If the "sensitivity" of the effector cell to acetylcholine does not change, the minimal effective concentration of acetylcholine will remain constant. The decrease in threshold associated with the decrease in the ChE activity indicates that a greater proportion of the acetylcholine liberated by frequencies previously subthreshold is now enabled to reach the effector cell in an amount equivalent to the minimal effective concentration. From this it follows that the amount of acetylcholine liberated by a threshold stimulus is in excess of the minimal effective concentration and that the total is in part inactivated by ChE before it can reach the effector cell. The cholinergic effects of DFP on resting cells thus

can be accounted for by the summation of constantly liberated, otherwise subliminal, amounts of acetylcholine.

In a similar manner the potentiation of the total response to maximal stimulation can be considered to result from the maintenance of an effective concentration of acetylcholine at the effector cell. Maximal shocks delivered at an appropriate frequency evoke a maximum secretory response which is consistent with the assumption that the receptors of the effector cell are at that time occupied fully. This is in accord with the observations that anti-ChE compounds do not potentiate the effects of maximal pre-ganglionic volleys. The reduction of the ChE activity results, then, in a prolongation of secretion rather than an enhancement of the response during stimulation. This latter fact affords, in addition, presumptive evidence that DFP does not alter the sensitivity of the cell. The increase in the post-stimulation secretion reaches its maximum at a ChE level of 10 per cent; further reduction of ChE produces a sudden sharp inflection in the response, which can be accounted for as a summation of stimulus effect and spontaneous activity. The potentiation of the total secretion of the salivary gland by DFP is analogous to the potentiation of the response of skeletal muscle to maximal shock after treatment with DFP. The latter also may be considered to be the result of summation of repetitive activity.

The sharp increase in the response to electrical stimulation or to a fixed dose of acetylcholine, when the ChE activity is reduced to 10 per cent or less, may represent a summation of the effects of the fixed stimulus and spontaneous neural activity. This becomes highly probable with the onset of spontaneous secretion, and finds support in the studies of Roeder *et al.* (17), who demonstrated in the isolated nerve cord of the cockroach, after treatment with DFP, a prolonged after-discharge of post-ganglionic fibres associated with bursts of spontaneous activity.

It can be postulated that the response of any cholinergic effector will be increased after appropriate amounts of DFP as a result of a summation of responses. The degree to which the ChE activity of the particular system will have to be inactivated to achieve this effect will depend upon the amounts of acetylcholine which that system is able to produce. For example, the amounts of acetylcholine liberated by chorda stimulation are large (5–10), in contrast to the exceedingly minute amounts which can be demonstrated in the perfusate of stimulated cat skeletal muscle (20). In accord with this the ChE activity of cat skeletal muscle must be inhibited almost completely before potentiation occurs (21). As a corollary the level of ChE at which spontaneous activity begins will depend likewise upon the acetylcholine production of the system.

The results obtained in this investigation provide certain pharmacologic criteria for the use of anti-cholinesterase compounds. From the data it is presumed that the progressive irreversible inactivation of tissue ChE will proceed in the manner described by equation I; the value of k_1 will be determined by the compound employed and the sensitivity of the ChE concerned. The therapeutic value of anti-ChE compounds is limited by the degree to which ChE must be inhibited before physiologic function is affected. If the therapeutic effect is to

be achieved by ChE inhibition alone, the level to which the enzyme must be reduced to affect a particular function will be the same regardless of the compound employed. With systemic administration the effect of the inhibitor cannot be confined to the enzyme of a particular tissue and a concomitant inactivation of other tissue ChE will proceed. Under these circumstances the margin of safety will be small. In this regard, a dose of 1 mgm./kgm. of DFP intravenously in the cat is without effect on the salivary gland ChE but is accompanied by minor toxic manifestations. This is also evident from the reports of Grob *et al.* (22, 23) who found that intramuscular doses of DFP sufficient to affect intestinal motility in man, when repeated daily for 2 to 3 doses, produced symptoms referable to the central nervous system associated with increased electroencephalographic activity. Finally, demonstrable changes in neuromuscular function were obtained in man only with comparatively large intra-arterial doses of DFP (24). The intravenous doses of DFP which would be necessary to achieve the latter effect would result in severe systemic signs. From the present study it is concluded that the optimal margin of safety will prevail when the desired site of action is a neuro-effector with a comparatively large acetylcholine potential so that function will be altered at relatively high levels of ChE activity. The ideal anti-ChE compound would be one with a selective affinity for a specific enzyme at a particular locus. These criteria are not satisfied by the agents available at present.

SUMMARY

The relationship between the inhibition of the cholinesterase (ChE) activity of the submaxillary gland and its physiologic responses is reported. The rate of irreversible inactivation of the ChE by DFP follows the characteristics of a first order reaction. Approximately 50 per cent of the glandular ChE can be inhibited without affecting response to nerve stimulation. From this point changes in function accompany a further progressive inactivation of ChE activity. These changes include a decrease in the frequency of stimulation required to elicit a threshold response, a prolongation of the response to maximal stimuli, and eventually the onset of spontaneous salivary secretion which begins at a ChE activity of 10 per cent of control.

The findings are discussed with respect to the physiologic and pharmacologic inter-relationships affecting chemical mediation at neuro-effector junctions.

ACKNOWLEDGEMENT. The authors wish to express their appreciation for the technical assistance of Miss Vivian L. Beach.

REFERENCES

1. MAZUR, A., AND BODANSKY, O.: J. Biol. Chem., **163**: 261, 1946.
2. KOELLE, G. B., AND GILMAN, A.: THIS JOURNAL, **87**: 421, 1946.
3. NACHMANSOHN, D., AND FELD, E. A.: J. Biol. Chem., **171**: 715, 1947.
4. BULLOCK, T. H., GRUNDFEST, H., NACHMANSOHN, D., ROTHENBERG, M. A., AND STERLING, K.: J. Neurophysiol., **9**: 253, 1946.
5. VON BEZNAK, A.: Pflüg. Arch. Physiol., **229**: 719, 1932.
6. GIBBS, O. S., AND SZELOCZEY, J.: Arch. Exper. Path., **168**: 64, 1932.

7. Babkin, B. P., Gibbs, O. S., and Wolff, H. G.· Arch. Exper. Path., 168: 32, 1932.
8. Babkin, B. P., Alley, A., and Stavraky, G. W.: Trans. Roy. Soc. Can., 26: 89, 1932.
9. Henderson, V. E., and Roepke, M. H.: This Journal, 47: 193, 1933.
10. Cattell, McK., Wolff, H. G., and Clark, D.: Am. J. Physiol., 109: 375, 1934.
11. Beznak, M., and Farkas, E.: Quart. J. Exper. Physiol., 26: 265, 1937.
12. Maltesos, C., and Weigmann, R.: Pflüg. Arch. Physiol., 241: 641, 1938.
13. Clark, A. J.: General Pharmacology. Julius Springer, Berlin, 1937.
14. Brassfield, C. R., Biggins, C., and Musselman, M. M.: Fed. Proc., 6: 80, 1947.
15. Riker, W. F., and Wescoe, W. C.: This Journal, 88: 58, 1946.
16. Hunt, C. C.: This Journal, 91: 77, 1947.
17. Roeder, K. D., Kennedy, N. K., and Samson, E. A.: J. Neurophysiol., 10: 1, 1947.
18. Grob, D., Lilienthal, J. L., Jr., Harvey, A M., and Jones, B. F.: Bull. Johns Hopkins Hosp., 81: 217, 1947.
19. Comroe, J. H., Jr., Todd, J., and Koelle, G. B.: This Journal, 87: 281, 1946.
20. Dale, H. H., Feldberg, W., and Vogt, M.: J. Physiol., 86: 353, 1936.
21. Hunt, C. C.: Unpublished observations.
22. Grob, D., Lilienthal, J. L., Jr., and Harvey, A. M.: Bull. Johns Hopkins Hosp., 81: 245, 1947.
23. Grob, D., Harvey, A. M., Langworthy, O R., and Lilienthal, J. L., Jr.: Bull. Johns Hopkins Hosp., 81: 257, 1947.
24. Harvey, A. M., Lilienthal, J. L., Jr., Grob, D., Jones, B. F., and Talbot, S. A.: Bull. Johns Hopkins Hosp., 81: 267, 1947.

INDEX